

Neurotoxins Affecting Neuroexocytosis

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Schiavo, Giampietro, Michela Matteoli, and Cesare Montecucco. Neurotoxins Affecting Neuroexocytosis. *Physiol. Rev.* 80: 717–766, 2000.—Nerve terminals are specific sites of action of a very large number of toxins produced by many different organisms. The mechanism of action of three groups of presynaptic neurotoxins that interfere directly with the process of neurotransmitter release is reviewed, whereas presynaptic neurotoxins acting on ion channels are not dealt with here. These neurotoxins can be grouped in three large families: 1) the clostridial neurotoxins that act inside nerves and block neurotransmitter release via their metalloproteolytic activity directed specifically on SNARE proteins; 2) the snake presynaptic neurotoxins with phospholipase A₂ activity, whose site of action is still undefined and which induce the release of acetylcholine followed by impairment of synaptic functions; and 3) the excitatory latrotoxin-like neurotoxins that induce a massive release of neurotransmitter at peripheral and central synapses. Their modes of binding, sites of action, and biochemical activities are discussed in relation to the symptoms of the diseases they cause. The use of these toxins in cell biology and neuroscience is considered as well as the therapeutic utilization of the botulinum neurotoxins in human diseases characterized by hyperfunction of cholinergic terminals.

I. INTRODUCTION

With the general aim of increasing their chance of survival, many thousands of living species produce toxins that are used to modify the physiology of other species. Toxins can be of any chemical complexity from very simple molecules, such as the formic acid used by ants, to the multimillion-dalton protein toxins produced by several bacteria. Some toxins are rather unspecific, but many of them are specific for a selected target molecule. It is conceivable that the specificity of certain toxins has been progressively refined during the course of evolution to alter the function of a selected target molecule, thus attaining specific goals within the strategy of survival of the toxin producer. Although most plant toxins are used for defense, animal toxins can be used for defense or predation of other animals or for both roles. Some bacterial toxins are directed against competing bacteria, whereas other toxins alter the physiology of the animal host to increase multiplication and/or diffusion of toxicogenic bacteria. Being the product of a long-term coevolution of the toxin-producing species with the target species, a toxin has very frequently been shaped around the target; hence, the study of its mechanism of action can reveal important features of host physiology (495).

In this light, it is not surprising that most known toxins are selective for molecules of the nervous tissue. All the most poisonous toxins are neurotoxins. Given the essential role of the nervous system in animal physiology, even a minor biochemical modification of a few neurons may result in a profound modification of behavior. Neurotoxins have played, and will play without doubt, a major role in unravelling nerve physiology (245, 257, 620).

In general, neurotoxins block in one way or another the transmission of the nerve impulse. A variety of animal neurotoxins act postsynaptically. They bind the acetylcholine receptor, the acetylcholinesterase or ion channels thereby blocking or altering their function. The majority of neurotoxins act presynaptically by binding specifically to ion channels, and the ensuing strong alteration of the permeability of the neuronal plasma membrane to selected ions results in an indirect inhibition of neuroexocytosis and in the blockade of the transmission of nerve signals. Neurotoxins that act simply by binding to neuronal molecules, thereby altering their physiological activity, are not dealt with in the present review, which focuses on the structure and mechanism of action of three groups of presynaptic neurotoxins that interfere directly and specifically with neurotransmitter release. Previous reviews have analyzed structural and functional aspects of these neurotoxins (149, 245, 419, 440, 509, 527, 620) as well as electrophysiological, ultrastructural, and molecular aspects of neuroexocytosis (48, 125, 590, 620). This review aims to provide an analysis of the mode of action of neurotoxins directly altering neuroexocytosis, in rela-

tion to the recent knowledge acquired on the synaptic vesicle exocytosis and endocytosis cycle.

II. EXO-ENDOCYTOSIS OF SYNAPTIC VESICLES

Transmission of a nerve muscle impulse follows a pre-synaptic depolarization that causes the opening of voltage-gated Ca^{2+} channels. This leads to a very rapid local increase of Ca^{2+} , up to 200 mM, which triggers, within 200–300 ms, the fusion of small synaptic vesicles (SSV) bound to specialized “active” zones of the presynaptic membrane (12, 180, 230, 291, 350, 493, 620). The synchronous release of these ACh quanta causes a large postsynaptic depolarization, termed end-plate potential (EPP). The resting neuromuscular junction (NMJ) spontaneously releases quanta of ACh, each of which is contained in a single small synaptic vesicle having a diameter of 40–50 nm (Fig. 1). This release causes a postsynaptic depolarization, termed miniature end-plate potential (MEPP) (291). Occasionally, giant MEPP can be observed. They account for 1–3% of the total number of synaptic events and correspond to a large Ca^{2+} -independent discharge of ACh, since the amount released is sufficient to activate the muscle fiber (296, 345, 597, 599). It has been suggested that giant MEPP derive from the release of ACh contained in endosomal compartments precursors of the SSV (35) or as a result of repair processes at damaged neuronal terminals (484, 599). After release, the SSV undergo rapid reuptake in a dynamin-dependent process and are refilled with neurotransmitter by proton-driven neurotransmitter transporters (Fig. 1) (48, 125, 138, 284, 590).

An extraordinary amount of research with the convergence of all experimental approaches presently available has focused on the identification and on the structural and functional characterization of the proteins involved in the SSV life cycle. This has led to the understanding that a very similar set of proteins and lipids is involved in all cellular events involving membrane fusion between a vesicular compartment and its target membrane (216, 346, 437, 514). Furthermore, these studies demonstrate that additional proteins are essential to sustain the unique features of neuroexocytosis, which is the most tightly regulated of such membrane trafficking events (48). In section IIK, we limit the discussion to an introduction of the family of proteins, known as SNARE (514), which are the target of the action of clostridial neurotoxins (CNT), and we refer the reader to recent reviews (48, 125, 587, 662) for synaptic proteins not included here.

III. NEUROTOXINS WITH METALLOPROTEASE ACTIVITY (CLOSTRIDIAL NEUROTOXINS)

A. Tetanus and Botulism

Eight neurotoxins endowed with a metalloprotease activity have been characterized so far, and the consequences

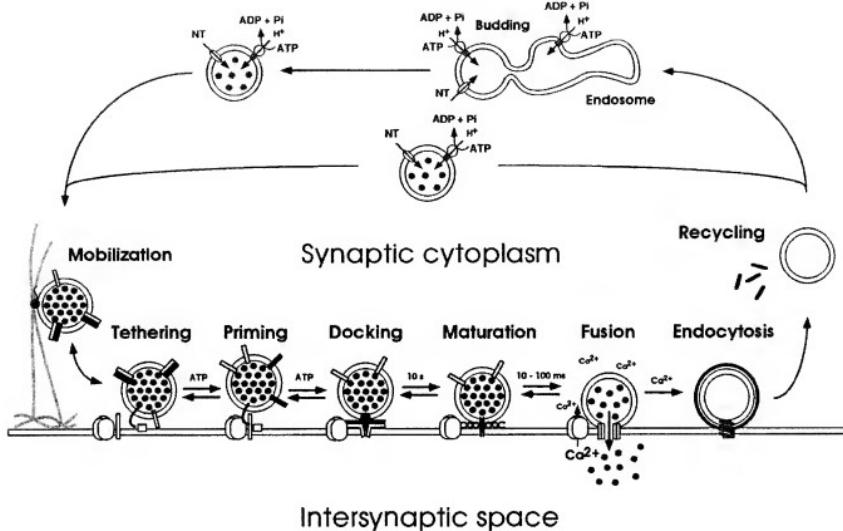


FIG. 1. The exocytosis-endocytosis cycle of synaptic vesicles at nerve terminals. Neurotransmitters (NT) are accumulated in the lumen of synaptic vesicles via specific vesicular transporters in a process driven by the pH gradient generated by the vacuolar ATPase proton pump (*top*). Most synaptic vesicles present in a typical synaptic terminal are bound to the actin cytoskeleton via interactions regulated by phosphorylation of proteins such as the synapsins (black comma on *left*). A small proportion of synaptic vesicles binds to the cytosolic face of the presynaptic membrane at active zones, via protein-protein interactions. Biochemical steps of this process have not been clarified, and the following part of the scheme is hypothetical and based on work performed mainly with systems not strictly related to the synapse such as the granule exocytosis in chromaffin cells and mast cells and the homotypic membrane fusion of yeast vacuoles. The binding process may involve a first phase of tethering, which implicates rab proteins and may be followed by priming catalyzed by cytosolic proteins, including N-ethylmaleimide-sensitive factor (NSF) and synaptosomal-associated proteins (SNAP) and the hydrolysis of ATP. Completion of the priming step leads to stabilization of the binding by additional protein-protein interactions involving a set of SNARE (docking), which form a *trans*-SNARE complex between the vesicle-associated membrane protein (VAMP) of the docked vesicle and 25-kDa SNAP (SNAP-25) and syntaxin, present on the cytosolic face of the presynaptic membrane. Docked vesicles may then become ready to bind Ca^{2+} and to fuse with the plasmalemma in a maturation step. Fusion is very rapidly triggered by the local increase of Ca^{2+} concentration that follows the opening of Ca^{2+} channels, located within the active zone. At the neuromuscular junction, the release of the ACh, contained inside one vesicle, causes a miniature end-plate potential, whereas the release of several vesicles corresponds to an end-plate potential. Exocytosis is rapidly ($<1\text{ s}$) followed by endocytosis in a process dependent on the formation of a clathrin coat and of a GTP-dependent action of dynamin. After pinching off the membrane, the coated vesicle uncoats and another cycle starts again.

of the activity of one of them (tetanus neurotoxin) have been known since the very beginning of medical literature. In fact, it was Hippocrates who described 25 centuries ago the symptoms of a paralyzed patient with hypercontracted skeletal muscles (358). He termed such a spastic paralysis tetanus (tetanos in greek means contraction). Tetanus is often fatal. Death follows body exhaustion and occurs by respiratory failure or heart failure (70). Tetanus still takes hundreds of thousands of lives per year, concentrated in those parts of the world where antitetanus vaccination is not compulsory

(198). This disease was thought to be of nervous origin until it was shown to be caused by a bacterium (97), which was isolated, characterized, and termed *Clostridium tetani* by Kitasato (301). This name derives from the elongated shape of the bacterium, which frequently harbors a subterminal spore, thus resembling a drum stick (clostridium in latin). *Clostridium tetani* is strictly anaerobic because it does not possess the redox enzymes necessary to reduce oxygen. Thus, in the presence of oxygen, radicals accumulate and lead eventually to bacterial death. *Clostridium tetani* is

widespread in nature in the form of spores, which germinate under appropriate conditions of very low oxygen tension, slight acidity, and availability of nutrients (481). Such conditions may be present in anaerobic wounds or skin ruptures or abrasions (even minor ones such as those caused by piercing or tattooing), where spores can germinate and produce a protein toxin that fills the bacterial cytosol and is released by autolysis. The toxin, termed tetanus neurotoxin (TeNT), is responsible for all of the symptoms of tetanus (169, 301, 602, 603).

Adult botulism was first recognized and described much later than tetanus (294), and infant botulism was described only 20 yr ago (17, 400, 473). This later recognition of botulism is to be attributed to the much less evident symptoms with respect to those of tetanus. In fact, botulism is characterized by a generalized muscular weakness, which first affects ocular and throat muscles and extends later to the whole skeleton (249, 522, 576). Botulism is likely to be much more frequent than can be deduced from the number of officially recorded outbreaks, because a partial muscular flaccidity may be considered not relevant enough to be reported (576, 621). In the more severe forms, a generalized flaccid paralysis accompanied by impairment of respiration and of autonomic functions develops, and death may result from respiratory failure (249, 576). Massive fatal outbreaks of botulism are not infrequent in animals, particularly among birds and fishes, both in the wild and on farms (576).

Botulism is caused by intoxication with one of the seven neurotoxins produced under anaerobic conditions by toxicogenic strains of *Clostridium botulinum* (621) or *Clostridium barati* and *Clostridium butyricum* (22, 235). The seven serotypically distinct botulism neurotoxins (BoNT) are indicated with letters from A to G. The spores of the different toxicogenic *Clostridia* sp. germinate under different conditions, and the bacteria differ for nutrient and temperature requirements (481). Such differences in growth conditions explain why, contrary to tetanus, botulism very rarely follows wound infection with spores of *C. botulinum* (wound botulism) (249). Usually, a BoNT is introduced by eating foods contaminated by spores of *C. botulinum* and preserved under anaerobic conditions that favor germination, proliferation, and toxin production (249, 522, 576). Similarly to most known proteins, BoNT are sensitive to the proteolytic and denaturating conditions found in the stomach lumen. It is believed that, to overcome this difficulty, they are produced as complexes with other nontoxic proteins (280, 402), which enable a proportion of BoNT to reach the intestine undamaged. Here, the slightly alkaline pH causes dissociation of the toxin complexes. BoNT could then reach general circulation by transcytosis from the apical to the basolateral side of intestinal epithelial cells (359) or by uptake from the M cells. There is evidence that in humans this process may be inefficient: as many as 10^6 mouse

LD_{50} of BoNT/A per milliliter of stool have been found in children showing moderate botulism symptoms during a recent outbreak of botulism in Italy (23) (P. Aureli, personal communication).

At the present time, we cannot exclude that part of the toxic complex is adsorbed as such in the oral cavity and/or in the esophagus and/or the stomach and that dissociation of the BoNT from the nontoxic proteins takes place in the circulating fluids. Alternatively, the complex may dissociate at early stages, and BoNT can be adsorbed in the first portions of the alimentary tract. In this case, one could hypothesize that the nontoxic proteins are made to preserve the BoNT from proteolytic attack in the bacterial culture medium.

As a consequence of the fact that a single protein is responsible for all the clinical symptoms of tetanus and botulism, these diseases can be completely prevented by antitoxin specific antibodies (198, 398, 399). Toxin-neutralizing antibodies can be acquired passively by injection of immunoglobulins isolated from immunized donors or, actively, as a result of vaccination with tetanus toxoid. The toxoid is obtained by treating TeNT or BoNT with paraformaldehyde (198, 494). Tetanus toxoid is very immunogenic and is used as a standard immunogen in a variety of immunological studies (123). More recently, antitetanus and antibotulism vaccines have been developed by genetic engineering techniques employing the COOH-terminal third of the TeNT or BoNT molecules (399). The general population is not vaccinated against botulism, since the disease is rather rare in developed countries, but vaccination may be performed on people involved in manipulation of toxicogenic *Clostridia* or of large quantities of BoNT. Vaccination does not appear necessary for scientists working with BoNT; the avoidance of using sharp objects, such as needles, and the availability in the laboratory of antisera anti-BoNT appear to be sufficient safety measures. The only known case of laboratory intoxication with BoNT occurred in workers attempting to administer an aerosol of BoNT/A to animals (268).

B. Presynaptic Activity of Clostridial Neurotoxins

The mouse LD_{50} values of TeNT and BoNT are between 0.1 and 1 ng toxin/kg body wt. Thus they are the most toxic substances known. Such values are expected to be even lower in the wild, where even a very small deficit in mobility may be sufficient to impair survival. Different animal species show a great range of sensitivity to TeNT and to the different BoNT. Humans and horses are at least as sensitive to these neurotoxins as mice, whereas rats, birds, snakes, and amphibians are rather resistant to TeNT, and turtles are insensitive (211, 460). The recent, ever-growing, use of BoNT/A as a therapeutic

agent for a variety of dystonias and other diseases has uncovered significant variations in the response of patients to the same dose of BoNT/A, with some individuals being unresponsive. The absolute neurospecificity of TeNT and BoNT and their catalytic activity (see below) are at the basis of such high toxicity. The time of onset of paralysis of animals injected with these neurotoxins is variable depending on species, dose, and route of injection. However, a lag phase, ranging from several hours to days, is always present between the time of injection and the appearance of symptoms. Of course, the lag phase is much longer when the disease is caused by contamination of wounds with spores of toxicogenic *Clostridia* because in this case the time of germination and bacterial proliferation to be included. The lag phase of tetanus in humans may be longer than 1 mo.

After entering the general circulation, CNT bind very specifically to the presynaptic membrane of motoneuron nerve endings. TeNT also binds to sensory and adrenergic neurons. Presynaptic receptor(s) have not been identified, but CNT are expected to bind rapidly and with high affinity to account for the limited spreading around the site of injection, experienced in clinical treatments, and for the low LD₅₀ values. After binding to the presynaptic membrane, the BoNT enter the neuronal cytosol and block the release of ACh, thus causing a flaccid paralysis (87, 387, 570). TeNT also binds to the motoneuron presynaptic membrane, but its peripheral action is zero or very limited, unless very high doses are injected (370). Contrary to BoNT, TeNT is transported retrogradely inside the motoneuron axon, in a microtubule-dependent movement, up to the spinal cord, where it accumulates in the ventral horn of the gray matter (85, 167, 186, 231, 238, 490, 588, 615). An intra-axonal ascendant transport rate of 7.5 mm/h has been estimated (588). Neuromuscular stimulation enhances the extent of uptake of CNT (233, 270, 319, 479, 644). Within the spinal cord, TeNT migrates *trans-synaptically* from the dendrites of peripheral motoneurons into coupled inhibitory interneurons across the synaptic cleft (547, 548), and it blocks the release of inhibitory neurotransmitters (40, 47, 82, 83, 129). Excitatory synapses appear not to be affected at early stages (46, 47, 82, 83, 390, 643, 646, 654), but they may be inhibited at later stages (596). This specificity of TeNT for inhibitory versus excitatory synapses is maintained when TeNT is applied to hippocampal slices (92) or injected into the hippocampus (389, 393). Such specificity for inhibitory synapses of the central nervous system (CNS) also accounts for the neurodegenerative and epileptogenic effects of TeNT, which mainly result from unopposed release of glutamate from excitatory synapses (24–26, 339). The selective action of TeNT on inhibitory synapses within the spinal cord may be at least in part due to the anatomical organization of the tissue because it is not preserved in spinal cord neurons in culture (47, 654).

During *trans-synaptic* migration, TeNT can be neutralized by antitoxin antibodies injected in the spinal fluid (166).

The blockade of inhibitory synapses brought about by TeNT at the spinal cord impairs the neuronal circuit that ensures balanced voluntary muscle contraction, thus causing the spastic paralysis characteristic of tetanus (390, 570, 643, 646). The half-life of ¹²⁵I-TeNT in the rat spinal cord and in cells in culture is several days (231, 234, 368). Such a figure compares well with the documented fact that tetanus symptoms may develop more than a month after wound infection, when the wound may have already healed. The amount of toxin that reaches the CNS, after uptake at the parasympathetic nervous system, is clearly an important parameter that determines the severity of the disease and may partly account for the different toxicity of TeNT in different vertebrates (460). Hence, the opposite clinical symptoms of tetanus and botulism result from different sites of action of TeNT and BoNT, rather than from a different mechanism of action (see also sect. III*L*). This neat distinction between the central site of activity of TeNT and the peripheral sites of action of the BoNT exists only at subpicomolar concentrations. To rapidly obtain consistent effects, hundreds of mouse lethal doses are frequently used in the laboratory, particularly when insensitive animals such as birds or fishes are studied or *in vitro* with cultured cells or isolated hemidiaphragm muscle preparations. Under such conditions, TeNT also inhibits peripheral synapses causing a botulism-like flaccid paralysis (370). In any case, CNT only act presynaptically causing a persistent inhibition of the exocytosis of a variety of neurotransmitters (reviewed in Ref. 643).

The action of TeNT and BoNT can be extended to a variety of nonneuronal cells by microinjection or addition to permeabilized cells: these neurotoxins then inhibit many, but not all, exocytotic events in a wide range of cells (6–8, 10, 29, 53, 57–59, 77, 108, 136, 137, 196, 197, 212, 226, 266, 278, 306, 307, 335, 377, 405, 454, 468, 500, 520, 584, 586, 592, 664).

C. Structure and Electrophysiology of Synapses Intoxicated With Tetanus and Botulinum Neurotoxins

Contrary to what has been seen with the animal neurotoxins described in sections IV and V, morphological examinations of synapses intoxicated *in vivo* or *in vitro* with CNT does not reveal major alterations of structure (Fig. 2). Synapses are not swollen; mitochondria, SSV, and large electron-dense vesicles are well preserved in terms of number, size, and intraterminal distribution. The only consistent change is an increase in the number of synaptic vesicles close to the cytosolic face of the presynaptic membrane (155, 156, 271, 320, 388, 434, 450, 462, 489). The BoNT/A poisoning of the frog NMJ causes the disappear-

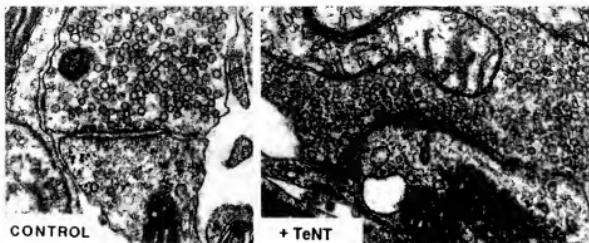


FIG. 2. The nerve terminal poisoned by tetanus neurotoxin (TeNT). Electron micrographs of control (*left*) and TeNT-treated dissociated spinal cord neurons (100 ng/ml for 10 h) (*right*) are shown. Notice the increase of synaptic vesicles juxtaposed to the presynaptic membrane of the intoxicated and electrically silent synapse. (Photos courtesy of Dr. E. A. Neale, National Institutes of Health, Bethesda, MD.)

ance of the small membrane invagination normally detectable close to the active zones, which are believed to represent SSV fusion events (491).

The first electrophysiological investigation of the effect of a BoNT on a NMJ was conducted by Burgen et al. (87) on the rat hemidiaphragm preparation. Following this seminal study, the consequences of CNT poisoning have been studied on different synaptic terminals, but only for the vertebrate NMJ is a large set of data available to compare the effects of TeNT and of BoNT. Most of these studies have been recently reviewed (96, 419, 484, 620). They can be summarized here as follows. 1) Clostridial neurotoxins cause a large and persistent blockade of EPP, responsible for the impaired synaptic transmission at intoxicated synaptic terminals; *in vitro*, on isolated neuronal cells in culture, they were shown to be effective on any synapse tested. 2) These neurotoxins greatly reduce the frequency, but not the amplitude, of evoked MEPP. Hence, CNT lower the number of vesicles capable of undergoing fusion and release, without affecting the ACh quantum. 3) CNT do not interfere with the processes of neurotransmitter synthesis, uptake, and storage (reviewed in Ref. 223). 4) TeNT and BoNT affect neither the propagation of the nerve impulse nor Ca^{2+} homeostasis at the synaptic terminal (152, 224, 361, 410). 5) The frequency of spontaneous MEPP is reduced, but not abolished, at poisoned terminals, and the neurotransmitter released during such residual events tends to be less and delivered more slowly. 6) The frequency of giant MEPP is not altered or even increases at the intoxicated NMJ (296, 408, 554, 555, 597). The effect of BoNT/C has been investigated only at CNS synapses with results comparable to those obtained with BoNT/A (96), whereas no report is available for BoNT/G. Clearly, more studies are necessary to provide a solid scientific basis for the clinical use of these neurotoxins. Meanwhile, BoNT/C has been proven to be as valuable as BoNT/A in the therapeutic treatment of human dystonias (165).

Based on the available data, CNT can be divided into two groups. BoNT/A and /E poison the NMJ in such a way that the quantal release of ACh evoked by nerve stimula-

tion remains synchronous. On the other hand, TeNT, BoNT/B, /D, and /F cause a desynchronization of the quanta released after depolarization (51, 154, 202, 241, 410). Aminopyridines, by inhibiting potassium channels, indirectly cause an increase in the Ca^{2+} level of the synapse and synchronize evoked neurotransmitter release in BoNT/A and /E poisoned terminals, leaving the release largely asynchronous in NMJ treated with TeNT and BoNT/B, /D, and /F (408, 484, 553, 643). Similar conclusions were reached with Ca^{2+} ionophores. An increased Ca^{2+} concentration within the synaptic terminal partially reverses the effect of BoNT/A and BoNT/C, is poorly effective on BoNT/E, and has no effect on TeNT-treated preparations (18, 29, 96, 128). A difference in the mechanism of action of these two groups of neurotoxins is also suggested by experiments of double poisoning of the NMJ with a CNT, followed by α -latrotoxin (LTX), which causes a massive release of SSV (see sect. v). α -Latrotoxin counteracts the action of BoNT/A, but not that of TeNT or BoNT/B (202).

These extensive electrophysiological studies led to several clear conclusions with which recent molecular data have to be compared: 1) CNT hit on synaptic terminal components playing essential roles within the neuroexocytosis machinery; 2) the CNT fall into two groups having different targets within the presynaptic terminal. On one side there are TeNT, BoNT/B, BoNT/D, and BoNT/F, and on the other side there are BoNT/A, /C, and /E; this conclusion is now fully substantiated by the identification of the molecular targets of each CNT (see sect. m); 3) The neurotoxin-impaired neuroexocytosis apparatus can still mediate some spontaneous residual synaptic activity, but with reduced efficiency with respect to the amount of neurotransmitter released and the rate of the overall process; 4) Giant MEPP occur via a mechanism not involving the CNT targets. Giant MEPP may be considered as indicators of immature or pathological states of the synapse, such as those occurring after tetanic stimulation or α -LTX-induced stimulation. It has been proposed that they result from a constitutive, rather than highly

regulated, type of exocytosis of ACh-containing endosomal compartments precursors of the SSV (35, 554).

D. Structure

The similar effect of the eight CNT at nerve terminals is the result of a closely related protein structure. They are synthesized in the bacterial cytosol without a leader sequence, which is in keeping with the fact that they are released in the culture medium only after bacterial lysis. No protein is associated with TeNT, whereas BoNT are released in the form of multimeric complexes, with a set of nontoxic proteins coded for by genes adjacent to the neurotoxin gene; these complexes are termed progenitor toxins (280, 402). Some BoNT-associated proteins have hemagglutinating activity (HA): HA of 17 kDa (HA17), HA of 34 kDa (HA34), and HA of 71 kDa (HA71). In addition, a large nontoxic nonhemagglutinating protein of 139 kDa (NTNH), coded for by a gene upstream to the BoNT gene, is always present. The NTNH produced by the various neurotoxicogenic strains of *Clostridia* are more conserved than the corresponding BoNT themselves. Moreover, a remarkable feature uncovered by gene sequencing is that the 100-amino acid-long NH₂-terminal region of NTNH is homologous to the corresponding region of BoNT (402). The significance of such an homology is unclear at the present time. It is tempting to suggest that the NH₂-terminal regions of BoNT and NTNH are independent domains with a strong tendency to dimerize. As such, they nest the formation of the BoNT-NTNH complex, which may then, or may not, progress to the formation of larger complexes. In fact, three forms of progenitor toxins have been characterized: extra-large size (LL sediments at 19S, ~900 kDa), large size (L sediments at 16S, 500 kDa), and medium size (M sediments at 12 S, 300 kDa). An electron-density projection map of the 19S complex of BoNT/A shows a triangularly shaped protein complex with six lobes (89).

The molecular genetics of CNT are currently under investigation, and several remarkable features are becoming apparent. They are beyond the scope of this review, and the reader is referred to recent reviews (402, 481). One general point has been firmly established: the neurotoxin genes are mobile, and nontoxicogenic strains cocultivated with toxicogenic strains can become toxicogenic by gene transfer mediated by phages or plasmids or conjugation transposons. Such processes are believed to occur during the enormous proliferation of anaerobic bacteria that takes place on animal cadavers converted by death into effective anaerobic fermentors. As a result of such genetic mobility, *C. botulinum* may harbor more than one toxin gene (275), and strains producing mosaic BoNT with type C and type D mixed elements have been recently characterized (428, 429).

Botulism neurotoxin in the form of progenitor toxins is more stable than isolated BoNT to proteolysis and denaturation induced by temperature, solvent removal, or acid pH (110, 522). Progenitor toxins that survive the harsh conditions of the stomach reach the intestine, where the slightly alkaline pH induces their dissociation and releases the BoNT, which is then transcytosed to the mucosal side of the intestinal epithelium (359). The inactive single-chain 150-kDa neurotoxins are activated by specific proteolysis within a surface-exposed loop subtended by a highly conserved disulfide bridge (Fig. 6). Several bacterial and tissue proteinases are able to generate the active dichain neurotoxin (130, 131, 318, 641). The heavy chain (H, 100 kDa) and the light chain (L, 50 kDa) remain associated via noncovalent protein-protein interactions and via the conserved interchain SS bond, whose integrity is essential for neurotoxicity (144, 537).

The length of the polypeptide chains of CNT varies from the 1,251 amino acid residues of *Clostridium butyricum* BoNT/E to the 1,297 residues of BoNT/G and the 1,315 residues of TeNT (402, 439). The exact length of the L and H chains depends on the site of proteolytic cleavage within the exposed loop. The L chains range in size from the 419 amino acid residues of BoNT/E to the 449 residues of TeNT. The H chains vary in size from the 829 amino acid residues of BoNT/E to the 857 residues of TeNT. As more and more amino acid sequences of BoNT are determined, it appears that their subdivision into seven serotypically distinct types is not adequate to describe their diversity. Very relevant sequence variations are present within the same BoNT serotype, and type C and type D hybrid toxin have been described (428, 429).

These polypeptide chains present homologous segments separated by regions of little or no similarity. The most conserved portions of the L chains are the NH₂-terminal 100 residues, mentioned above, and the central regions (residues 216–244, numbering of TeNT). Eight NH₂-terminal residues and 65 COOH-terminal residues can be deleted from TeNT without loss of activity (322). The 216–244 region contains the His-Glu-Xaa-Xaa-His binding motif of zinc-endopeptidases (286, 287, 322, 538, 540, 614, 663). This observation led to the demonstration that CNT are zinc proteins (535, 538–540, 542, 543, 668). One atom of zinc is bound to the L chain TeNT, BoNT/A, /B, and /F (538, 540, 543) with a dissociation constant (K_d) value in the 50–100 nM range, at the lower limit of the known range of affinities among metalloproteases. Flow dialysis also showed multiple zinc binding sites with lower affinity (540, 663). Heavy metal chelators remove bound zinc and generate inactive aponeurotoxins (265, 538, 571), without appreciable changes in L chain secondary structure (139). The active site metal atom can be reacquired upon incubation in zinc-containing buffers to reform the active holotoxin (265, 535, 538, 540, 542, 543, 571). With the same procedure, the active site zinc atom

can be exchanged with other divalent transition metals forming active metal-substituted toxins (604).

The crystallographic structures of BoNT/A and the COOH-terminal TeNT domain (H_C) have been recently determined at 3- and 1.61-Å resolution, respectively (304, 326, 611). The toxin structure reveals three distinct functional domains, a unique hybrid of previously characterized structural motifs, and new insight into this protein's mechanism of toxicity (Fig. 3). There is complete agreement with the three-domain structural model of CNT previously proposed to account for the available biochemical data (418). BoNT/A consists of three ~50-kDa domains: an NH_2 -terminal domain endowed with zinc-endopeptidase activity; a membrane translocation domain characterized by the presence of two 10-nm-long α -helices, which are reminiscent of similar elements present in colicin and in the influenza virus hemagglutinin; and a binding domain composed of two unique subdomains similar to the legume lectins and Kunitz inhibitor (326).

Such structural organization is functionally related to the fact that CNT intoxicate neurons via a four-step mechanism consisting of 1) binding, 2) internalization, 3) membrane translocation, and 4) enzymatic target modification (417, 419). The L chain is responsible for the intracellular catalytic activity (10, 57, 58, 407, 468, 486, 640). The NH_2 -terminal 50-kDa domain of the H chain (H_N) is implicated in membrane translocation (69, 151, 201, 264, 412, 561), whereas the COOH-terminal part (H_C) is mainly responsible for the neurospecific binding (61, 238, 430, 642).

The H_C domains of TeNT and BoNT/A are very sim-

ilar with an overall elongated shape (Fig. 4), and preliminary data on the crystallographic structure of BoNT/E reveal a closely similar organization (R. C. Stevens, personal communication). The H_C domains of the two BoNT appear to be very flexible with respect to the H_N domain. The binding domains of these three CNT consist of two distinct subdomains, the NH_2 -terminal half (H_C-N) and the C-terminal half (H_C-C), with little protein-protein contacts among them. H_C-N has two seven-stranded β -strands arranged in a jelly-roll motif closely similar to that of legume lectins, which are carbohydrate binding proteins. The amino acid sequence of this subdomain is highly conserved among CNT, suggesting that it has a closely similar three-dimensional structure in all the CNT. The H_C-C contains a modified β -trefoil folding motif present in several proteins involved in recognition and binding functions such as interleukin-1, fibroblast growth factor, and Kunitz-type trypsin inhibitors. Its sequence is poorly conserved among CNT. Removal of H_C-N from H_C does not reduce H_C nerve membrane binding, whereas deletion of only 10 residues from the COOH terminus abolishes its binding to spinal cord neurons (237). The critical importance of the last 34 residues of H_C-C , and in particular of His-1293 of TeNT, for binding the oligosaccharide portion of polysialogangliosides was recently demonstrated by photoaffinity labeling (556). These data are supportive of a double receptor model of binding of CNT to the presynaptic membrane (413) (see sect. III G for a discussion) with H_C-N binding to a glycoprotein, different for the different CNT, and H_C-C binding to a polysialoganglioside, whose

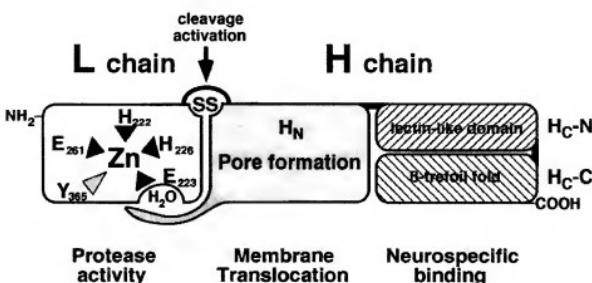


FIG. 3. Three domain structure of clostridial neurotoxins. These neurotoxins consist of 3 domains of similar size (50 kDa). NH_2 -terminal domain (left) is a zinc endopeptidase, which is inactive when disulfide bonded to the rest of the molecule; its activity is expressed after reduction of the interchain disulfide bond. The active site zinc atom is coordinated by 2 histidine residues, a water molecule bound to a conserved glutamate residue and by the carboxylate group of another glutamate, with the likely participation of a conserved tyrosine (residue numbering corresponds to TeNT). H_N , the central domain, is responsible for the membrane translocation of the L chain into the neuronal cytosol. The COOH-terminal H_C domain (right) consists of two equally sized subdomains. The NH_2 -terminal subdomain has a structure similar to that of sugar binding proteins. The COOH-terminal subdomain folds similarly to proteins known to be involved in protein-protein binding functions such as the K^+ channel specific dendrotoxin. Such structure is consistent with the toxin binding to the presynaptic membrane via a double interaction, most likely with two different molecules of the nerve terminal.

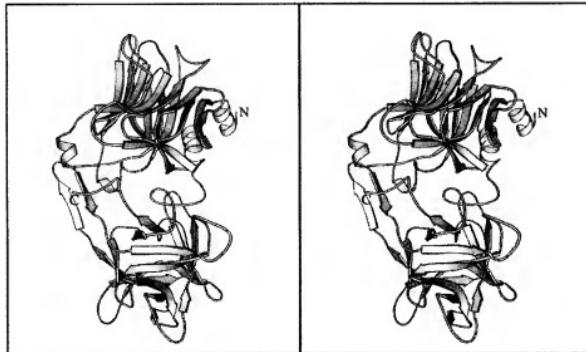


FIG. 4. Stereo-pair view of the receptor binding domain of tetanus neurotoxin. COOH- and NH₂-terminal ends of the fragment H_c of TeNT are labeled, and these correspond to residues Glu-875 and Asp-1315, respectively, of the intact neurotoxin. The first 11 residues at the NH₂ terminus were disordered in the crystal structure and hence are not displayed here. Residues Glu-875 to Ser-1110 form a subdomain possessing a jelly-roll folding motif, and this fold is similar to that displayed by the legume lectins (*top*). Residues Ile-1111 to Asp-1315 form the second subdomain of the H_c fragment, containing the β -trefoil motif, similar to Kunitz-type protease inhibitors and to dendrotoxin (*bottom*). For further explanations, see text. (Photo courtesy of Dr. T. C. Umland.)

nature may be similar for the various CNT. Additional toxin-membrane protein interactions cannot be excluded. The major difference between H_c-C of TeNT and BoNT/A resides in the structure of the loops, thus suggesting that these external segments may be responsible for the binding to the different protein receptors.

The H_N portions are highly homologous among the various CNT (402), and their predicted secondary structure is also highly similar (337). The membrane translocation domain of BoNT/A has a cylindrical shape determined by the presence of a pair of unusually long and twisted 10-nm-long α -helices, corresponding to segment 685–827, reminiscent of the α -helices hairpin of colicin (652). At both ends of the pair there is a shorter α -helix that lies parallel to the main helices and, in addition, several strands pack along the two core helices. It is difficult to identify the residues and segments involved in the formation of ion channel at low pH, but the overall structure of H_N resembles that of some viral proteins that undergo an acid-driven conformational change (86, 639). A remarkable feature of BoNT/A is an extended loop that wraps around the catalytic L chain and makes it difficult to account for the fact that a short incubation of BoNT/A with dithiothreitol is sufficient to release the L chain.

The catalytic metalloprotease domain, 55 Å × 55 Å × 62 Å, contains both α -helix and β -strand secondary structures and has little similarity with related enzymes of known structure, apart from the α -helix including the zinc binding motif (326). In addition to the imidazole rings of the two histidines of the motif and a water molecule bound to the glutamic acid of the motif, the zinc atom of BoNT/A is coordinated by Glu-261 and the phenolic ring of Tyr-365 points versus the metal atom, but remains ~5 Å away from it. This type of zinc coordination resembles that of thermolysin, but sequence differences as well as

the unique properties of metal substituted TeNT (604) and of the differences found in the multiple scattering analysis of the X-ray absorption spectra of TeNT in comparison with metalloproteases of known three-dimensional structure (425) clearly indicate that these neurotoxins have an active site of unique architecture. A recent biophysical analysis compared TeNT with astacitin and thermolysin and suggested that the phenolic ring of the active site Tyr residue of the isolated L chain of TeNT may be closer to the zinc atom than that indicated by the crystallographic structure of BoNT/A (394). Another characteristic of the active site of BoNT/A is that it is 20–24 Å deep in the protein and that it is accessible via an anionic channel, not accessible in the intact molecule because it is shielded by H_N and its wrapping belt (326). This accounts for the lack of enzymatic activity of dichain CNT. This active site channel becomes accessible to the substrate upon reduction of the interchain disulfide bridge and appears to be capable of accommodating 16 amino acid residues.

E. Neurospecific Binding

From the site of production or absorption, BoNT and Tent diffuse in body fluids and reach and bind to the presynaptic membrane of cholinergic terminals. Tetanus neurotoxin may also bind to sympathetic and adrenergic fibers (reviewed in Refs. 238, 643). The introduction of radiolabeling methods allowing the production of active CNT at highly specific activity has made possible binding studies with unprecedented and still unsurpassed sensitivity. For technical reasons, almost invariably, the binding of CNT to CNS acceptors present in particulate brain matter, isolated lipid preparations, or synaptosomes has been studied. Only more recently, isolated neuronal cells

in culture have been considered. These studies are hampered by the fact that all CNT lose some activity upon radiolabeling and that the range of concentration of clinical significance are undetectable with radioiodinated CNT. These extensive studies have not provided definitive answers with respect to affinity and number of binding sites of the individual CNT but have revealed heterogeneous binding of these neurotoxins to the presynaptic membrane with subnanomolar and nanomolar binding sites. These studies have been carefully reviewed before (27, 232, 238, 643) and are not dealt with here since the conclusion presented in Habermann and Dreyer (232) is still largely valid. [There is no doubt that specific binding sites do exist for botulinum neurotoxins (and tetanus neurotoxin) and probably even for the individual types with a varying degree of cross-reactivity. However, only a very small percentage of total binding is expected to display sufficient affinity, which is a prerequisite for the extreme potency of this toxin *in vivo* and on isolated organs.] Radiolabeled BoNT can be monitored also by autoradiographic methods, and it was thus possible to demonstrate that they bind to the unmyelinated zone of motoneuron terminals in a temperature-independent mode (150). Hundreds of binding sites for BoNT/A and /B per square millimeter appear to be present in the rat NMJ (63), whereas the number of TeNT receptors in a neuroblastoma-glioma cell line is ~450/cell (645). BoNT/A and BoNT/B also bind to cholinergic nerve terminals of rat brain, without apparent binding to other terminals (65).

Available evidence indicates that the H_c domain plays a major role in neurospecific binding (61, 115, 255, 312, 328, 430, 560, 642). However, it appears that additional regions of CNT are involved in binding because H_c shows only a partial protection from intoxication with the intact CNT molecule, and the H_c fragment of TeNT does not prevent retroaxonal transport of the holotoxin (61, 191, 407, 483, 487, 488, 595, 640). If binding is carried out above 20°C, the radiolabeled and fluorescent- or gold-tagged, CNT are internalized inside intracellular compartments of different nature after an energy-dependent endocytosis step (63, 126, 150, 375, 457, 458, 482).

Previous attempts at identification of the presynaptic receptor(s) of CNT have been reviewed before (for reviews and references, see Refs. 238, 390, 413, 414, 643). Here we only mention results that are relevant with respect to the recent identification of a sugar-binding subdomain in BoNT/A and TeNT (326, 610). Beginning with the seminal work of van Heyningen (622–624), a large number of studies have established that polysialogangliosides are involved in binding CNT (54, 126, 168, 230, 237, 267, 289, 299, 300, 369, 392, 421, 422, 446, 530, 533, 567, 589, 628, 630, 653, 671, 677). The results of these studies are briefly summarized hereafter: 1) CNT bind to polysialogangliosides, particularly to G_{D1b}, G_{T1b}, and G_{Q1b}; 2) preincubation with polysialogangliosides partially pre-

vents the BoNT poisoning of the NMJ and the retroaxonal transport of TeNT; 3) incubation of cultured cells with polysialogangliosides increases their sensitivity to TeNT and BoNT/A; and 4) treatment of membranes with neuraminidase, which removes sialic acid residues, decreases toxin binding. Binding to polysialogangliosides well accounts for an unsaturable low-affinity binding of the CNT to nerve cells and to nerve tissue membranes. However, as discussed in detail previously (390, 413), it is unlikely that polysialogangliosides are the sole receptors of these neurotoxins. Experiments carried out with cells in culture have indicated that proteins of the cell surface may be involved in toxin binding (458, 474, 534, 672). The sugar-binding and protein binding subdomains present in the H_c domain of TeNT and BoNT/A (326, 611) and the protection experiments mentioned above support the suggestion that CNT may bind strongly and specifically to the presynaptic membrane because they display multiple interactions with sugar and protein binding sites (413). Recent experiments provided strong evidence in favor of such a model by showing that BoNT/B binds strongly to the synaptic vesicle protein synaptotagmin II in the presence of polysialogangliosides and that Chinese hamster ovary cells transfected with the synaptotagmin II gene bind the toxin with low affinity and with a high affinity after membrane incorporation of gangliosides GT1b (441–443). More recently, BoNT/E was also reported to interact with synaptotagmin (343).

Generally, receptors for toxins and viruses are cell surface molecules essential for the life of the cell, and their study has led to important progresses in cell biology and neurosciences. The identification of the receptors of the CNT is particularly relevant for several theoretical and practical reasons. In fact, both TeNT and BoNT bind the presynaptic membrane of α-motoneurons, but then they follow different intracellular trafficking paths. The electrophysiological studies discussed above have clearly shown that BoNT block neuroexocytosis at peripheral terminals, whereas TeNT causes the same effect on CNS synapses of the spinal cord. These different final destinations of TeNT and BoNT must be determined by specific receptors that drive them to different intracellular routes. The determination of the nature of the peripheral motoneuron TeNT receptor(s) will uncover an entry gateway leading from the peripheral to the central nervous system. This is expected to help in devising novel routes to deliver biologicals, including analgesic and anesthetic agents, into the spinal cord. The knowledge of the receptors for the various BoNT will also contribute to improving present therapeutic protocols and may explain the lack of effect of BoNT/A in a subset of patients that do not benefit from the current BoNT/A treatment.

To reach its final site of action, TeNT has to enter inside two different neurons: a peripheral motoneuron and an inhibitory interneuron of the spinal cord. Its bind-

ing to peripheral and central presynaptic terminals is different, as indicated by several pieces of evidence. 1) Cats and dogs are highly resistant to TeNT administered peripherally but very sensitive to the toxin injected directly in the spinal cord (564). 2) The L-H_N fragment of TeNT injected in the cat leg is not toxic, whereas it causes a spastic paralysis upon direct injection into the spinal cord (595). It is possible that the concentration of TeNT in the limited space of the synaptic cleft between peripheral motoneuron and inhibitory interneuron is significantly higher than that at the periphery, with the motoneuron acting as a sort of "toxin pump." If this is the case, even a low-affinity receptor could mediate the entry of TeNT in the latter cells, because of its anatomically restricted location within the intersynaptic space. Lipid monolayer studies have clearly documented the ability of 10⁻⁸ M TeNT to interact with acidic lipids (533). Similar concentrations are routinely used with cells in culture and in hippocampal injections *in vivo* (391) or in experiments of induction of a flaccid paralysis in mice treated with 1,000 times the mouse LD₅₀ dose (370). On the other hand, in clinical tetanus and botulism, at the periphery, TeNT and BoNT act at subpicomolar concentrations. A possible scenario that reconciles the presently available data is summarized hereafter. Glycoprotein and glycolipid binding sites are implicated in the peripheral binding of CNT, which is characterized by high affinity and high specificity. The protein receptor of TeNT would be responsible for its inclusion in an endocytic vesicle that moves in a retrograde direction all along and inside the axon, whereas BoNT protein receptors would guide them inside vesicles that acidify within the NMJ. The TeNT-carrying vesicles reach the cell body and then move to dendritic terminals to release the toxin in the intersynaptic space. The TeNT equilibrates between pre- and postsynaptic membranes and then binds and enters the inhibitory interneurons via synaptic vesicle endocytosis.

Contrary to TeNT, there is no evidence that BoNT can reach the CNS in botulism patients. However, in rats injected with high doses of BoNT/A, a little fraction can reach the spinal cord (229, 650, 651), and cats in the lateral rectus muscle of the eye show some signs of central effects (427). On the other hand, BoNT/A does inhibit neuroexocytosis in isolated CNS preparations (reviewed in Ref. 643). Thus is possible that BoNT at high doses, like high doses of TeNT do, act on sites of the nervous system that are unaffected in clinical botulism.

F. Internalization Inside Neurons

Because the L chains of CNT block neuroexocytosis by acting in the cytosol, at least this toxin domain must reach the cell cytosol. All available evidence indicates that CNT do not enter the cell directly from the plasma

membrane, rather are endocytosed inside acidic cellular compartments. Electron microscopic studies have shown that, after binding, CNT enter the lumen of vesicular structures in a temperature- and energy-dependent process (62, 63, 126, 150, 375, 457, 583). The H_C domains of TeNT and BoNT/A, B, and E appear to be sufficient for the internalization process in murine spinal cord neurons (328). Montesano et al. (424) found TeNT inside non-clathrin-coated vesicles, but their study was performed on liver cells exposed to very large concentrations of TeNT. Gold-labeled TeNT was internalized by spinal cord neurons inside a variety of vesicular structures, and only a minority of TeNT was in the lumen of SSV (457). In contrast, Matteoli et al. (375) found TeNT almost exclusively inside small synaptic vesicles of hippocampal neurons after a 5-min membrane depolarization. It was long known that nerve stimulation facilitates intoxication (233, 270, 319, 479, 644). A prominent neuroexocytosis correlates with a high rate of synaptic vesicle recycling via endocytosis and refilling with neurotransmitter, being the two processes tightly coupled (48, 125, 549, 590). The simplest way to account for the shorter onset of paralysis induced by CNT under conditions of nerve stimulation is that the neurotoxins enter the synaptic terminal via endocytosis inside the lumen of SSV. Hippocampal neurons are the best available test system for such an hypothesis because 1) TeNT is active on the hippocampus, causing an epileptic-like syndrome when injected in this brain area (78, 207); 2) antibodies specific for epitopes of SSV luminal proteins bind to them during neurotransmitter release and are taken up inside the terminals after SSV endocytosis (316, 373, 432); 3) SSV endocytosis can be followed accurately with dyes such as FM1-43 (49, 50); 4) a high rate of SSV exo-endocytosis can be induced at synaptic terminals simply by briefly incubating the cells in a Ca²⁺-containing, high-potassium medium, using as a control a Ca²⁺-free medium; and 5) during their development, growing axons are characterized by a high rate of spontaneous SSV recycling (316, 374). Tetanus neurotoxin was found to enter synaptic terminals of hippocampal neurons inside the lumen of SSV (375). The toxin was also found to be internalized inside SSV spontaneously recycling in growing axons of hippocampal neurons. Similarly, TeNT enters inside granular cells of the cerebellum (O. Rossetto, P. Caccin, and C. Montecucco, unpublished results). These studies indicate that TeNT uses SSV as "Trojan horses" to enter inside CNS neurons. Similar experiments on peripheral motoneurons would permit the evaluation of such a possibility for BoNT at peripheral synapse, but it is presently difficult to maintain these cells in culture and to perform similar experiments.

As discussed above, TeNT and BoNT have to enter different vesicles at the NMJ to account for their different destiny inside peripheral motoneurons. Alternatively, they could enter inside the same vesicles with TeNT

causing a vesicle modification/lesion such that the TeNT-containing vesicle is induced to bind to the microtubule-dependent motor involved in retroaxonal transport. In contrast, the BoNT-containing vesicles would remain within the motoneuron presynaptic terminal. In this respect, it is noteworthy that BoNT can intoxicate CNS neurons only when present at high concentrations. A high concentration of BoNT appears to enter hippocampal neurons via the aspecific process of fluid-phase endocytosis (C. Verderio, S. Coco, A. Bacci, O. Rossetto, P. De Camilli, C. Montecucco, and M. Matteoli, unpublished observations), but it is possible that this is not the case for cholinergic CNS neurons.

G. Translocation Into the Neuronal Cytosol

Whatever the nature of the vesicles containing the internalized neurotoxins, the L chains must cross the hydrophobic barrier of the vesicle membrane to reach the cytosol where they display their activity. The different trafficking of TeNT and BoNT at the NMJ clearly indicates that internalization is not necessarily linked to, and followed by, membrane translocation into the cytosol, i.e., internalization and membrane translocation are clearly distinct steps of the process of cell intoxication, as is the case for most intracellularly acting bacterial toxins (396, 417). There is indirect, but compelling, evidence that TeNT and BoNT have to be exposed to a low pH step for nerve intoxication to occur (3, 375, 568, 569, 572, 566). Acidic pH does not induce a direct activation of the toxin via a structural change, since the introduction of a non-acid-treated L chain in the cytosol is sufficient to block exocytosis (10, 57, 58, 407, 468, 486, 640). Hence, low pH is instrumental in the process of membrane translocation of the L chain from the vesicle lumen into the cytosol. In this respect, TeNT and BoNT appear to behave similarly to the other bacterial protein toxins characterized by a structure consisting of three distinct domains (417). Low pH induces TeNT and BoNT to undergo a conformational change from a water-soluble "neutral" form to an "acid" form with surface-exposed hydrophobic segments, which enable the penetration of both the H and L chains in the hydrocarbon core of the lipid bilayer (73, 74, 91, 395, 420, 421, 504, 532). After this low pH-induced membrane insertion, TeNT and BoNT form ion channels in planar lipid bilayers (69, 73, 151, 201, 264, 395, 496, 546, 561). These ion-conducting channels are cation-selective with conductances of a few tens of picoSiemens and are permeable to molecules smaller than 700 Da. There is evidence that these channels are formed by the oligomerization of the H_N domain (151, 395, 546, 561). The structure of the H_N domain of BoNT/A has elements of similarity with other membrane translocating toxins such as colicins and diphtheria toxin, which make channels (112, 456, 652) and with some viral proteins of viruses undergoing low pH-driven structural

changes (174, 639). Site-directed mutagenesis coupled to electrophysiological investigations and biochemical studies of diphtheria toxin and colicins membrane insertion indicate that the hairpin pair of buried hydrophobic helices is the first part of the molecule that enters the lipid bilayer followed by other α -helices of the same domain (reviewed in Refs. 124, 415). Peptides corresponding to segment 668–690 of TeNT (GVVLLLEYIPEITLPIVIALSIA) and segment 659–681 of BoNT/A (GAVILLEIFIPEIAIPVLGTFALV), which are predicted to form amphipatic α -helices, but are actually β -stranded in the crystallographic structure neutral form (326), form channels with properties similar to those of the intact toxin molecule (412). On this basis, it was proposed that the channel is formed by a toxin tetramer that brings four amphipatic helices into proximity with the carboxylates of the two Glu residues of the segment pointing inside the channel (412). This is compatible with the three-dimensional image reconstruction of the channel formed by BoNT/B in phospholipid bilayers (546). Clostridial neurotoxin channel formation is not limited to model membranes, since TeNT forms ion channels in spinal cord neurons. They open with high frequency at pH 5.0, but not at neutral pH; are rather nonselective for Na⁺, K⁺, Ba²⁺, and Cl⁻; and have a single-channel conductance of 45 pS (39).

There is a general consensus that these toxin channels are related to the process of translocation of the L domain across the vesicle membrane into the nerve cytosol. However, there is no agreement on how this process may take place. According to one hypothesis, the L chain unfolds at low pH and permeates through a transmembrane pore formed by H chain(s). After exposure to the neutral pH of the cytosol, the L chain refolds, and it is released from the vesicle by reduction of the interchain disulfide bond (73, 264). In this "tunnel" model, the formation of a transmembrane ion-conducting pore is a prerequisite for translocation. Two experimental results do not fit in this model: 1) the L chains of TeNT and BoNT/A, B, and E penetrate the lipid bilayer in such a way as to be exposed to the fatty acid chains of phospholipids, i.e., they are not shielded from lipids inside the H chain tunnel (420, 421); and 2) values of the order of a few tens of picoSiemens do not account for the dimensions expected for a protein channel that has to accommodate a polypeptide chain with lateral groups of different volume, charge, and hydrophilicity. The protein-conducting channels of the endoplasmic reticulum, of *Escherichia coli* and of mitochondrial membranes, characterized in planar lipid bilayers, have a conductance of 220 pS (181, 261, 321, 565, 566, 600). These channels are closed when plugged by a transversing polypeptide chain. Changing the size or polarity of the applied voltage does not influence their conductance or gating, whereas it does affect CNT channels.

A second model, advanced by Beise et al. (39), envisages that as the vesicle internal pH decreases after the operation of the vacuolar-type ATPase proton pump, CNT

insert into the lipid bilayer, forming ion channels that grossly alter electrochemical gradients. Eventually, such permeability changes cause an osmotic lysis of the toxin-containing acidic vesicle, emphasized by possible toxin-induced destabilization of the lipid bilayer (91). The membrane barrier is broken, and the cargo of toxin molecules is released in the cytosol. Even though this model greatly simplifies the problems posed by membrane-translocating toxins, some experimental findings with diphtheria toxin, which provides the best-characterized system with respect to bacterial toxin entry into cells, do not support it.

- 1) Diphtheria toxin forms ion channels in the plasma membrane of living cells at low pH without causing cell lysis (11, 455, 526), and similarly, TeNT does not lyse the plasmalemma of neuronal cells at pH 5.0 (39).
- 2) Endosomes containing diphtheria toxin can be isolated from cells (38, 341).
- 3) A catalytically inactive diphtheria toxin mutant form alters the plasma membrane permeability to sodium and potassium without lysing the cell (455).
- 4) Diphtheria toxin that has not translocated in the cytosol moves further along the endosomal-lysosomal pathway to be eventually degraded (341, 453).

This osmotic lysis model could be tested directly by determining if fluid-phase markers gain access to the cytosol in the presence of toxins.

An alternative hypothesis, which explains all available experimental data, proposes that the L chain translocates across the vesicle membrane within a channel open laterally to lipids, rather than inside a proteinaceous pore (416, 417). The two toxin polypeptide chains are supposed to change conformation at low pH in a concerted fashion, in such a way that both of them expose hydrophobic surfaces and enter into contact with the hydrophobic core of the lipid bilayer. The toxin acid form may have the dynamic properties of a molten globule (90, 619). The H chain forms a transmembrane hydrophilic cleft that nests the passage of the partially unfolded L chain with its hydrophobic segments facing the lipids. The cytosolic neutral pH induces the L chain to refold and to regain its water-soluble neutral conformation, after reduction of the interchain disulfide. It is possible that cytosolic chaperones are involved in treadmilling the L chain out of the vesicle membrane and in assisting its cytosolic refolding, but as yet there is no supporting evidence. As the L chain is released from the vesicle membrane, the transmembrane hydrophilic cleft of the H chain is supposed to tighten up to reduce the amount of hydrophilic protein surface exposed to the membrane hydrophobic core. However, this leaves across the membrane a peculiarly shaped channel with two rigid protein walls and a flexible lipid seal on one side. This is proposed to be the structure responsible for the ion-conducting properties of TeNT and BoNT. In this "cleft" model, the ion channel is a consequence of membrane translocation rather than a prerequisite. Moreover, ion transport is mediated by a

transmembrane structure that derives from the one involved in the L-chain translocation, but which is physically different.

II. Zinc-Endopeptidase Activity

The catalytic activity of these neurotoxins was discovered following the sequencing of the corresponding genes, which began with TeNT (160, 173) and, within a few years, was extended to all CNT (402). Sequence comparison revealed a highly conserved 20-residue-long segment, located in the middle of the L chain, containing the His-Glu-Xaa-Xaa-His zinc-binding motif of zinc-endopeptidases (322, 538, 540, 663). Building on this observation, investigators soon demonstrated that TeNT inhibited ACh release at synapses of the buccal ganglion of *Aplysia californica* via a zinc-dependent protease activity (538). Identification of the cytosolic substrates of such enzymic activity followed assays of proteolysis performed on SSV and on other synaptic proteins suggested as candidates for the neuroexocytosis apparatus by the characterization of a 7S brain complex (579).

The eight CNT are remarkably specific proteases; among the many proteins and synthetic substrates assayed so far, only three of them, the so-called SNARE proteins, have been identified (Figs. 5 and 6 and Table 1). TeNT and BoNT/B, /D, /F and /G cleave vesicle-associated membrane protein (VAMP)/synaptobrevin, but each at different sites (531, 535, 538, 539, 543, 666, 667); BoNT/A and /E cleave 25-kDa synaptosomal-associated protein (SNAP-25) at two different sites and BoNT/C cleaves both syntaxin and SNAP-25 (56, 67, 68, 189, 450, 539, 541, 542, 655). Strikingly, TeNT and BoNT/B cleave VAMP at the same peptide bond (Gln-76-Phe-77), yet when injected in the animal, they cause the opposite symptoms of tetanus and botulism, respectively (531). This observation clearly demonstrated that the different symptoms derive from different sites of intoxication rather than from a different molecular mechanism of action of the two neurotoxins.

Recombinant VAMP, SNAP-25, and syntaxin are cleaved at the same peptide bonds as the corresponding cellular proteins, thus indicating that no additional endogenous factors are involved in determining the specificity of the CNT. It was recently reported that CNT are phosphorylated inside the neuron and that this modification enhances the proteolytic activity of the toxins as well as their lifetime inside the cytosol (178). These findings have been exploited to develop *in vitro* assays of the metalloprotease activity of CNT (162, 163, 236, 536, 577). Particularly useful will be continuous assays based on the use of fluorescent substrates, whose fluorescence is internally quenched and is freed upon proteolysis of the peptide bond that keeps the two fluorophores close to each other (305) (F. Cornille and B. P. Roques, unpublished observa-

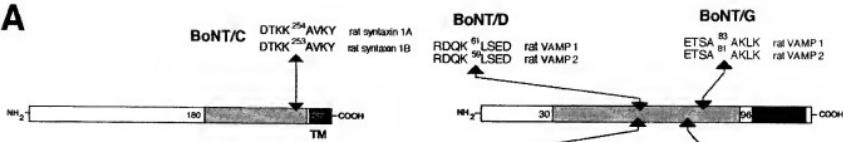
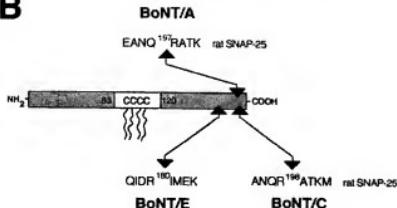
A**B**

FIG. 5. Schematic structure of syntaxin I and SNAP-25 with cleavage sites of clostridial neurotoxins. *A:* syntaxin is a type II membrane protein consisting of 4 parts: a NH₂-terminal region (1-180) that folds in a bundle of α -helices with a left-handed twist, followed by a region (180-262, gray box) that participates in SNARE complex formation via α -helix coiling around complementary regions of VAMP and SNAP-25. Botulinum neurotoxin (BoNT)C cleaves within this second part of syntaxin and compromises the functional pairing of the vesicle with the presynaptic membrane, thus preventing the ensuing vesicle-membrane fusion. The third part is a typical transmembrane segment (black box) followed by a short extracellular COOH-terminal segment. *B:* SNAP-25 lacks a classical transmembrane segment, and its membrane binding is mediated by the palmitoylation of a group of cysteines located in the middle of the polypeptide chain. Cleavage sites for BoNT/A, C, and E (arrows) and the 2 segments essential for the interaction with other SNARE (gray boxes) are indicated.

tions). The proteolytic activity of the CNT can be probed in cells and tissues with antibodies specific for epitopes present in the intact SNARE molecule, which are released into the cytosol following the action of the toxin. A highly sensitive single-cell assay can thus be performed by following the progressive loss of SNARE staining as its proteolysis progresses (375, 450, 492, 655). In parallel, the progressive block of SSV exo-endocytosis recycling consequent to substrate proteolysis can be monitored by assaying the internalization of antibodies specific for epitopes of SSV lumen (373).

Two groups of zinc-endopeptidase inhibitors are known: *1)* zinc chelators and *2)* molecules that bind with high affinity to the active site. Zinc chelators act either by complexing the free zinc that is in chemical equilibrium with the active site zinc, as EDTA does, or by actively removing the protein-bound metal atom, as *ortho*-phenanthroline does (21). The latter type of chelators is much more rapid and should be used when a quick inactivation of a zinc-endopeptidase is needed. Some zinc chelators are plasma membrane

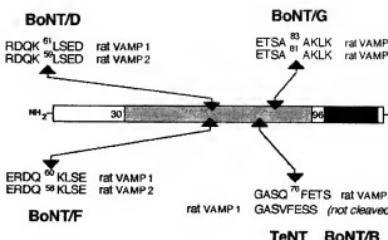


FIG. 6. Schematic structure of VAMP. VAMP/synaptobrevin is a type II membrane protein with a short COOH-terminal tail protruding in the vesicle lumen, a transmembrane segment (black box), followed by a 66-residue-long cytosolic part, which is highly conserved among isoforms and species (gray box). This central portion of VAMP coils around complementary regions of SNAP-25 and syntaxin in the SNARE complex and contains the site of interaction and cleavage by the clostridial neurotoxins. In contrast, the NH₂-terminal part is poorly conserved and rich in prolines and remains outside the SNARE complex. It is likely to be involved in protein-protein interactions with other components of the neuroendocytosis apparatus and transport proteins. The cleavage sites for TeNT, BoNT/B, D, F, and G are indicated by arrows. Rat VAMP-1 isoform is not cleaved by TeNT and BoNT/B, due to a sequence variation at the cleavage site, but this is not the case for other species, such as humans and mice.

permeable and can be used in intact cells (538). Although chelators are very effective on the CNT, none of the inhibitors active on the other classes of zinc-endopeptidases acts on CNT at low concentrations (120, 538) as a consequence of the different active site geometry of this novel group of zinc-endopeptidases. Specific inhibitors are badly needed not only for biochemical and cellular studies but also to evaluate them as potential drugs for the treatment of tetanus and botulism. Recently, a fluorescent coumarin derivative was found to inhibit BoNT/B (4), and a series of aminothiol derivatives of tripeptides next to the VAMP cleavage of TeNT and BoNT/B have been shown to inhibit in the high

TABLE 1. *Tetanus and botulism neurotoxins: target and peptide bond specificities*

Toxin Type	Intracellular Target	Peptide Bond Cleaved P ₁ P ₂ P ₃ P ₄ –P _{1'} P _{2'} P _{3'} P _{4'}
TeNT	VAMP	Gly-Ala-Ser-Gln-Phe-Glu-Thr-Ser
BoNT/A	SNAP-25	Glu-Ala-Ser-Gln-Ala-Thr-Lys
BoNT/B	VAMP	Gly-Ala-Ser-Gln-Phe-Glu-Thr-Ser
BoNT/C	Syntaxin	
BoNT/C	SNAP-25*	Asp-Thr-Lys-Ala-Ala-Val-Lys-Phe
BoNT/D	VAMP	Ala-Asn-Gln-Ala-Thr-Lys-Met
BoNT/D	SNAP-25	Arg-Gln-Lys-Lys-Leu-Ser-Glu-Leu
BoNT/F	VAMP	Gln-Ile-Asp-Arg-Ile-Met-Glu-Lys
BoNT/G	VAMP	Glu-Arg-Asp-Gln-Lys-Leu-Ser-Glu

TeNT, tetanus neurotoxin; BoNT, botulism neurotoxin; VAMP, vesicle-associated membrane protein; SNAP-25, 25-kDa synaptosomal-associated protein. * H. Niemann, personal communication.

micromolar range (366). Combinatorial chemistry is being applied on these derivatives, with some novel inhibitors acting in the low micromolar range (L. Martin, F. Cornille, S. Turcaud, H. Mendal, B. P. Roques, and M. C. Fournié-Zaluski, unpublished observations). It can be anticipated that novel powerful inhibitors will be designed on the crystallographic structure of BoNT/A, and the goal of having specific antitetanus and antibotulism therapeutic agents may soon be reached.

I. Targets of Clostridial Neurotoxins

1. Syntaxin

Syntaxin is a type II membrane protein of 35 kDa, located mainly on the neuronal plasmalemma (Fig. 5A) (33, 42, 44, 279). The NH₂-terminal portion is exposed to the cytosol and is followed by a transmembrane domain and few extracellular residues (42, 44). The cytosolic region is composed of two domains characterized by distinct structural features. The NH₂-terminal domain of monomeric syntaxin (residues 1–120) consists of three long α -helices, which are likely to be involved in protein-protein interactions (177), whereas the central portion (residues 180–262) enters in a four helix bundle structure upon interaction with the other members of the SNARE protein complex (477, 591) (see sect. III/4). A vast syntaxin polymorphism exists within the nervous tissue, and syntaxins constitute a large protein family with more than 20 isoforms in mammals and with homologs in yeast and plants (43, 71). In the active zones, syntaxin is associated with several types of Ca²⁺ channels (52, 132, 342, 367, 447, 558, 581, 582, 660, 673). Syntaxin is also present on chromaffin granules (594) and undergoes, together with SNAP-25, a recycling process in organelles indistinguishable from synaptic vesicles (629).

Syntaxin interacts with the other t-SNARE SNAP-25 and the v-SNARE VAMP/synaptobrevin to form a protein complex known as synaptic SNARE complex, which constitutes the core of the neuroexocytosis apparatus (579). SNARE complex formation is controlled by the interaction of syntaxin with Munc-18 (termed also n-Sec1 and rbsSec1), and it is regulated by protein kinase C (195, 263, 590). Syntaxin interacts in a Ca²⁺-dependent equilibrium with some isoforms of the synaptic vesicle protein synaptotagmin, the likely Ca²⁺ sensor in neurotransmitter release (590). The *Arabidopsis KNOLLE* gene encodes a protein related to syntaxin, which is specifically expressed during mitosis and is required for cytokinesis (355). A similar function has been revealed in *Drosophila*, where syntaxin 1 is essential for the cellularization of early embryos (88).

Syntaxins are important for neuronal development and survival, since BoNT/C, unlike the other CNT, acts as cytotoxic factor in neurons (323, 450, 657). Several isoforms, including syntaxins 1 and 3, undergo a com-

plex pattern of alternative splicing and expression during long-term potentiation, thus suggesting that syntaxins are involved in synaptic plasticity (260, 507, 517). This differential expression could be important for a direct modulation of Ca²⁺ entry via selective interaction with specific Ca²⁺ channels, in addition to the formation of distinct SNARE complexes with different SNAP-25 and VAMP isoforms.

2. SNAP-25

SNAP-25 is a major palmitoylated protein in the CNS (259, 451, 659). Because of the absence of a canonical transmembrane segment (Fig. 5B), its membrane localization is thought to be mediated by the palmitoylation of cysteine residues located in the middle of the polypeptide chain (259, 331, 625). SNAP-25 is conserved from yeast to humans (81, 659), with little variation in length and size. SNAP-25 self-associates to form a disulfide-linked dimer, both *in vitro* and *in vivo* (518). The SNAP-25 forms a stoichiometric complex with the putative Ca²⁺ sensor synaptotagmin, and this interaction is believed to be important for the Ca²⁺-dependent phase of neurotransmitter release (29, 544). Furthermore, SNAP-25 was demonstrated to interact in a Ca²⁺-dependent manner with Hrs-2, an ATPase having a negative regulatory effect on neuroexocytosis (37). SNAP-25 is required for axonal growth during neuronal development and in nerve terminal plasticity in the mature nervous system (206, 449). SNAP-25 is developmentally regulated, with the two isoforms A and B switching their expression in the nervous system and neuroendocrine cells at birth (30). The synthesis of both isoforms is upregulated in hippocampal neurons during long-term potentiation, thus suggesting their involvement in synaptic plasticity (506). Recently, SNAP-23, a SNAP-25 isoform expressed only outside the nervous system, was also identified (281, 411, 497, 519, 631). In some regulated secretory pathways, this isoform can replace SNAP-25, thus suggesting a partial overlapping in their functions (519). In mast cells, SNAP-23 relocates from the plasma membrane to the granule membrane in response to stimulation. After relocation, SNAP-23 is required for exocytosis, implying a crucial role of this SNAP-25 isoform in promoting membrane fusion (225). SNAP-29, a longer isoform of SNAP-25 with a conserved BoNT/E cleavage site, has been very recently cloned from a human library (585).

3. VAMP

VAMP (also referred to as synaptobrevin) is a protein of 13 kDa localized to synaptic vesicles, dense core granules, and synaptic-like microvesicles and is the prototype of the vesicular SNARE (v-SNARE) (44, 578, 590). Four functional domains can be distinguished in the VAMP molecule (Fig. 6) (36, 607). The NH₂-terminal 33-residue-

long part is proline rich and isoform specific, whereas the following region (residues 33–96) is very well conserved through evolution and contains coiled-coil regions and sites of phosphorylation for the Ca^{2+} /calmodulin-dependent protein kinase type II and the casein kinase type II (438). The protein is anchored to the synaptic vesicle membrane via a single transmembrane domain, which is followed by a poorly conserved intravesicular tail of variable length in different species. Recently, splicing variants of VAMP-1 with modified COOH-terminal sequences have been described (282, 363). In one case (VAMP-1B), the splicing process shortens the predicted transmembrane region by four residues and appends a functional mitochondrial localization signal to the COOH terminus of VAMP (282).

Ten different isoforms have been identified through database searches on the basis of structural sequence similarity (5, 71, 200, 661, 675), but only three isoforms of VAMP have been extensively characterized: VAMP-1, VAMP-2, and cellubrevin (36, 380, 607). The VAMP isoforms are present in all vertebrate tissues, but their relative amount and distribution differ (357, 511, 606). On the synaptic vesicle membrane, VAMP is associated with synaptophysin, a major component of SSV membrane and with subunits of the V-ATPase (93, 159, 199, 634). VAMP also interacts with VAP-33, a protein of unknown function, which is specifically localized on SSV in *Aplysia californica* (575) but has a much broader distribution in mammalian cells (638). VAMP-2, but not VAMP-1 or cellubrevin, interacts with a prenylated Rab acceptor via its proline-rich and its transmembrane segment as assessed by yeast two-hybrid screening and direct binding (363). The presence of isoform-specific VAMP/synaptobrevin binding proteins is confirmed by the isolation of BAP31, a sorting protein that controls the trafficking of VAMP-1 and cellubrevin, but not VAMP-2 (15). Very recently, functional evidence on the involvement of VAMP-2 also in SSV biogenesis was provided by the study of its interaction with the heterotetrameric adaptor complex AP3. In fact, the CNT-mediated ablation of VAMP-2 from endosomes blocks completely SSV formation *in vitro* and their coating with AP3 (524).

In *Caenorhabditis elegans*, the VAMP-homolog snb-1 is not essential for embryogenesis, but the animals die soon afterward because they are incapable of coordinated movements and, therefore, of feeding (445). This impairment is due to a very deficient neurotransmitter release, which is however not completely abolished. This result is confirmed in null VAMP mutants in *Drosophila* (140), which suggests that a portion of the spontaneous exocytic events that are seen at the synapse can be generated by a protein complex that is distinct from that required for an evoked synaptic response.

4. Properties of the synaptic SNARE complex

The three targets of the zinc-endopeptidase activity of CNT, VAMP, syntaxin, and SNAP-25 are largely unstructured as recombinant proteins in solution but form a heterotrimeric complex characterized by a high structural stability (252). Very recent structural studies (477, 591) have shown that the SNARE complex consists of four tightly packed α -helices, wrapped in parallel around each other to form a quadruple left-handed helical bundle. This rod-shaped bundle retains the membrane-anchoring sequences at one end of the rod and adopts a geometry already noticed in several membrane fusion segments of viral glycoproteins, such as gp41s from human and simian immunodeficiency virus and in the low-pH conformation of the influenza (574). This quadruple helical bundle is believed to constitute the core of the SNARE complex and derives from the association of most of the cytoplasmic domain of synaptobrevin (residues 30–96), the COOH-terminal portion of the cytoplasmic domain of syntaxin (residues 180–262), and the NH_2 - and COOH-terminal segments of SNAP-25 (residues 1–83 and 120–206). SNAP-25 contributes two parallel α -helices, i.e., oriented in the same way, linked by a long extended segment, which includes the quartet of palmitoylated Cys residues, implying that the SNARE complex lies parallel to the membrane surface. These regions were previously highlighted in deletion studies on individual SNARE proteins (252, 292), with the exception of SNAP-25 whose NH_2 -terminal portion only appeared to be required (252, 292).

The NH_2 termini of VAMP (residues 1–27) and of syntaxin (residues 1–120) do not take part in the formation of the four helix bundles and constitute two cytoplasmic extensions of the SNARE complex. The proline-rich NH_2 -terminal of VAMP is clearly implicated in exocytosis, since its removal inhibits the process (55), and NH_2 -terminal peptides inhibit neurotransmitter release (119). The native SNARE complex, which presents a sedimentation coefficient of 7S, can recruit in suitable conditions other cytosolic proteins, shifting its sedimentation coefficient to 20S. These factors are *N*-ethylmaleimide-sensitive factor (NSF) and its soluble adaptors soluble NSF accessory proteins (SNAP). NSF as well as the SNAP were initially purified as cytosolic factors required for the reconstitution of intra-Golgi transport *in vitro* (513, 514, 578, 662), and they are both recognized as essential proteins for a large number of vesicular transport steps within the cell. NSF is an hexameric ATPase that catalyzes the disassembly of the SNARE complex into monomeric components (648, 662). The accumulation of vesicles in an *in vitro* intra-Golgi assay after NSF depletion, in yeast NSF mutant sec18, and in the correspondent comatose mutant in *Drosophila* suggested the possibility that NSF could be involved in docking and fusion of the transport vesicles (452, 514, 578). Recent experiments using a lysosome

homotypic fusion assay in yeast indicate that the action of NSF may be restricted to an earlier stage, the predocking and/or docking step (228, 376, 612). Recent experiments with permeabilized adrenal and pheochromocytoma cells also support this possibility, although a role for NSF in a postdocking stage of heterotypic membrane fusion cannot be excluded at the present time (251, 550).

The 20S synaptic SNARE complex is stable in the presence of nonhydrolyzable ATP analogs but is rapidly disassembled in the presence of ATP and Mg²⁺ (31, 240, 579). Very recently, the low-resolution structure of the 20S particle has been determined with electron microscopic and rotary shadowing techniques (240). In the 20S particle, NSF and SNAP occupy one end of the rod that constitutes the α -helical core of the SNARE, and they disappear when the complex is incubated in the presence of Mg²⁺-ATP (240). The structure and properties of the SNARE complex and of its components suggest that complex formation brings the membranes anchoring the SNARE in close proximity. The free energy released during complex formation may be at least partially used to promote membrane bilayer fusion (503). Such a model is supported by the finding that synthetic liposomes with reconstituted VAMP can interact and fuse in vitro with vesicles containing reconstituted full-length SNAP-25 and syntaxin 1 (637). This model would also require dissociation of the stable complex at some subsequent stage during the vesicle recycling process; indeed, NSF and α -SNAP, in the presence of ATP, can make the stable complex susceptible to dissociation (253).

Several other synaptic proteins have been shown to interact with the isolated SNARE proteins and with the complex (346). Their role is not clear, but it is likely that they regulate the formation and rate of assembly/disassembly of the membrane fusion machinery as well as controlling the sorting of individual molecules during vesicle endocytosis.

The effects of CNT on assembly and disassembly of the stable SNARE complex support the idea that a cycle of assembly and disassembly is a key process in exocytosis. In fact, cleavage of individual SNARE by CNT does not prevent SNARE complex formation, but either this association is less stable (252, 465) or it loses its functional connection to the membrane by the result of affecting a step of the neuroexocytosis process that occurs after the formation of the highly stable form of the SNARE complex.

In contrast, SNARE are resistant to CNT when assembled in the SNARE complex (252, 464). The transition from total cleavability of the SNARE proteins in the isolated form to the complete resistance upon entry in the complex is consistent with the gain in secondary structure experienced by the SNARE during complex formation. In fact, VAMP and SNAP-25 are largely unstructured as monomers (175), whereas they are α -helical within the SNARE complex (477,

591). It is a well-established notion that peptide bonds included in α -helices are highly resistant to proteolytic cleavage (188), and together with the possible inaccessibility of the motifs acting as secondary CNT recognition sites (see section mK), this is sufficient to account for this resistance. However, the completeness of the inhibition of vesicle fusion by most of CNT suggests that their target proteins spend long periods of their lifetime in a nontoxin-sensitive state. In the case of BoNT/E-mediated cleavage of SNAP-25 in chromaffin cells, this sensitive period extends even past the ATP-dependent priming step, which includes dissociation of SNARE complex by NSF and α -SNAP, supporting the notion that the VAMP, SNAP-25, and syntaxin are stably engaged in the complex only in a late stage in the fusion process (29, 210, 664).

J. SNARE Cleavage and Neurotransmitter Release Inhibition

Several experimental data indicate that there is a direct correlation between neurotoxin-induced proteolysis of VAMP or SNAP-25 or syntaxin and inhibition of neurotransmitter release

The SNARE proteins are cleaved in synaptosomes and cells intoxicated with TeNT or BoNT with a corresponding inhibition of exocytosis (3, 67, 68, 96, 189, 265, 335, 348, 375, 450, 454, 492, 500, 520, 539, 655).

The intracellular activity of the toxins is inhibited, although at high concentrations, by specific inhibitors of zinc-endopeptidases such as phosphoramidon and captopril (136, 143, 538).

Peptides spanning the cleavage site of VAMP inhibit TeNT and BoNT/B in *Aplysia* and squid neurons or chromaffin cells (136, 271, 538).

A VAMP-specific antibody prevents the inhibition of neurotransmitter release in *Aplysia* neurons induced by TeNT and BoNT/B, but not that caused by BoNT/A (485).

TeNT-resistant mutants of VAMP (499) and BoNT/A-resistant mutants of SNAP-25 (P. Washbourne, N. Bortolotto, M. E. Graham, M. C. Wilson, R. D. Burgoyne, and C. Montecucco, unpublished observations) restore exocytosis in intoxicated cells.

An antibody against the zinc-binding segment inhibits the activity of TeNT and BoNT/A in chromaffin cells (34).

In synapses of invertebrates and vertebrates, inhibition of neurotransmitter release induced by the CNT is paralleled by the cleavage of the corresponding a SNARE protein (84, 258, 271, 492).

In isolated hippocampal neurons, cleavage of VAMP by TeNT or BoNT/B is accompanied by a large inhibition of SSV recycling (C. Verderio, S. Cocco, A. Bacci, O. Rossetto, P. De Camilli, C. Montecucco, and M. Matteoli, unpublished observations).

Taken together, these findings provide direct evi-

dence for the involvement of VAMP, SNAP-25, and syntaxin in exocytosis in general. At the same time, they provide a molecular understanding of the pathogenesis of tetanus and botulism based on the cleavage of SNARE proteins. Another general conclusion of these studies is that full inhibition of neurotransmitter release is not accompanied by a parallel full proteolysis of the SNARE proteins present within a nerve terminal (84, 189, 450, 492, 655). On the contrary, only a partial proteolysis is seen both by immunofluorescence and by immunoblotting. This result is best explained by the existence within the nerve terminal of different pools of SNARE proteins with different availability to binding and proteolysis by the CNT. A large proportion of a SNARE protein may be involved in protein-protein interactions or may be physically segregated in such a way that the protease cannot degrade it. On the other hand, it appears that those SNARE molecules that are engaged or are about to be engaged in neuroexocytosis are available for the proteolysis by the CNT, and it is their cleavage that results in the block of neurotransmitter release.

The catalytic intracellular activity of these neurotoxins greatly contributes to their potency. In fact, inside a synaptosomal terminal, one L chain is expected to cleave one after another all substrate molecules present therein. It can be estimated that in *Aplysia* neurons 4–10 molecules of TeNT L chains are sufficient to inhibit 50% of neuroexocytosis within 20 min at 20°C (B. Poulain, personal communication). Considering the higher body temperature of mammals and the length of the onset of tetanus and botulism symptoms in humans, it would not be surprising if, given the due time, a single toxin molecule is sufficient to fully intoxicate one synapse. This explains the well-documented clinical finding that tetanus symptoms can develop even after a month from the healing of the skin wound contaminated by *C. tetani* spores.

K. Specificity for VAMP, SNAP-25, and Syntaxin

An inspection of the nature and sequence of amino acid residues at and around the cleavage sites of the various CNT for the three SNARE proteins (Table 1) reveals no conserved patterns accounting for the target specificity of these metalloproteases. Hence, each neurotoxin must differ in the detailed spatial organization of the active site, to accommodate the SNARE segment to be cleaved and to catalyze the hydrolysis of specific and different peptide bonds. Biochemical studies have uncovered several peculiarities of these metalloproteases. 1) Short peptides encompassing the cleavage site are not cleaved, although they bind the toxin, as deduced by their inhibition of the toxin action in *Aplysia* neurons and in neurohypophysis (136, 531, 538, 562). 2) However, peptides corresponding to longer segments of the substrate

proteins are cleaved (119–121, 190, 562, 563, 577, 666). 3) Although TeNT and BoNT/B hydrolyze the same peptide bond of VAMP, the minimal VAMP segment cleaved is peptide 44–94 in the case of BoNT/B and peptide 33–94 in the case of TeNT (190). 4) Some BoNT hydrolyze a peptide bond, while leaving intact other peptide bond(s) of the same type located in another part of the substrate molecule. More precisely, a) BoNT/D cleaves the Lys-59-Leu-60 peptide bond, but not the Lys-83-Leu-84 peptide bond, of rat VAMP (539); b) BoNT/G cleaves rat VAMP-2 at the Ala-81-Ala-82 peptide bond and leaves intact the Ala-5-Ala-6 bond (535, 666); c) BoNT/A cleaves SNAP-25 at the Gln-197-Arg-98 peptide bond but does not hydrolyze the Gln-15-Arg-16 peptide bond within the same molecule (56, 541); d) BoNT/E cleaves the Arg-180-Ile-181 peptide bond, but not the bond between Arg-59 and Ile-60, of SNAP-25, (56, 541); e) BoNT/C cleaves syntaxin Ia at the Lys-253-A-254 peptide bond and does not affect the Lys-260-Ala-261 bond of syntaxin (542); and f) moreover, the same toxin cleaves SNAP-25 at the Arg-254-Ala-255 bond and not at the Arg-17-Ala-18 bond. 5) BoNT/C only cleaves membrane-bound SNAP-25 and syntaxin and is ineffective on the isolated molecules (68, 542); also, other neurotoxins are more effective on the membrane-bound substrate than on the recombinant soluble molecule (450, 467, 655).

These findings clearly indicate that CNT recognize the tertiary, rather than the primary, structure of their three proteolytic substrates. Analysis of their primary and secondary structure (337, 402) suggests that these neurotoxins are structurally very similar. At the same time, the variable cleavage sites and flanking regions do not account for the specificity of the CNT. These considerations prompted a search of the sequences of SNARE proteins involved in neuroexocytosis that led to the identification of a nine-residue-long motif, termed thereafter SNARE motif (512). This motif is characterized by the presence of three carboxylate residues alternated with hydrophobic and hydrophilic ones. The motif is always contained within regions predicted to be α -helical and, consequently, the three negatively charged residues cluster on one face adjacent to an hydrophobic face. There are two copies of the motif in VAMP (V1 and V2) and syntaxin (X1 and X2) and four copies in SNAP-25 (S1, S2, S3, and S4). Several pieces of experimental evidence support this proposal. 1) Only those protein segments including at least one SNARE motif are cleaved (120, 122, 190, 562, 577). 2) The motif is exposed on the protein surface as shown by binding of anti-SNARE motif antibodies. These antibodies cross-react among the three SNAREs and inhibit the proteolytic activity of the neurotoxins (467). 3) The various neurotoxins cross-inhibit each other (467). 4) Proteolysis performed on site-directed mutated VAMP or VAMP fragments indicate that the three carboxylate residues of V2 are very important for the recognition by BoNT/B and /G, whereas those of the V1 copy of the motif are implicated

in recognition of BoNT/F and of TeNT (466, 467, 563, 649). BoNT/D shows a particular requirement for the Met-46 present in V1 (466, 666). These results explain why the minimal length of VAMP segments cleaved by TeNT is longer than that required by BoNT/B (190, 562), since they have to include V1, which is more NH₂-terminal with respect to V2. Because of the similarity between TeNT and BoNT/B, these results also suggest the possibility that the two copies of the SNARE motif of VAMP are paired in such a way that they adopt the same spatial orientation with respect to the Gln-76-Phe-77 bond (467). In addition, a basic region located after the cleavage site of TeNT and BoNT/B is important for their binding, and optimal cleavage of VAMP (119, 122, 562, 666) has provided evidence that TeNT may behave in this respect as an allosteric enzyme activated by binding to these two regions of the substrate that are external to the cleaved region. 5) The SNARE motif is also important for binding and proteolysis of SNAP-25 by BoNT/A and /E. The analysis of the rate of proteolysis of several SNARE motif-deleted SNAP-25 fragments shows that they are hydrolyzed, provided that at least one of the four copies of the motif is retained. In other words, the four copies of the SNARE motif can largely substitute for one another with respect to recognition and proteolysis by BoNT/A and /E (633). This result indicates a large flexibility of SNAP-25, which is not surprising for a molecule that has to interact in a reversible way with partner molecules of the neuroexocytosis apparatus (107, 252, 253, 465).

These experiments are necessarily performed with recombinant, soluble substrates, and an extrapolation of what happens *in vivo* on the endogenous membrane-bound molecules is not straightforward.

Taken together, these studies suggest that a major determinant of the specificity of the CNT for the three SNARE proteins is the recognition of the SNARE motif. This is followed by further interaction with regions, located in different parts of the sequence, that are specific of each SNARE; they include the segment containing the peptide bond to be cleaved as well as other segments (see the model depicted in Fig. 7). The relative contribution of these multiple interactions to the specificity and strength

of neurotoxin binding to each SNARE protein remains to be determined. However, it can be predicted that hydrolysis of the substrate region bound to the active site of the neurotoxin causes a decrease in the binding affinity, which is the result of a multivalent type of interaction (Fig. 7). Cleavage is expected to lead to a rapid release of the two fragments.

The regions of TeNT and BoNT involved in substrate binding are unknown. It is tempting to suggest that the strongly conserved 100-residue-long NH₂-terminal region is involved. Removal of more than eight residues from the NH₂ terminus leads to complete loss of activity (322). This region includes a segment (80–100) predicted to be α -helical (337). It is noteworthy that this segment is rich in positively charged residues that would lie on the same face of the helix and could interact electrostatically with the negative charges of the SNARE motif.

Additional biological activities of CNT have been reported. TeNT was shown to be capable of binding and activating synaptic transglutaminases (TGase), and synapsin was found to be an excellent TGase substrate (20, 170, 171). Synapsin is involved in a phosphorylation-dependent linkage of SSV to the actin cytoskeleton (616), and it was suggested that toxin-activated TGase cross-links synaptic vesicles to synapsin, thus rendering them unavailable to exocytosis. This would cause a long-term inhibition of SSV exocytosis superimposed to the rapid inhibition due to VAMP proteolysis (170, 172). This proposal has been challenged by Coffield et al. (116), who found no evidence for an involvement of a TGase in TeNT action. More recently, Regazzi et al. (499) have shown that a TGase-mediated activity of TeNT is not involved in the toxin inhibition of the exocytosis of insulin-containing vesicle. Furthermore, active site mutants of TeNT and BoNT/A, devoid of metalloprotease activity, are unable to inhibit ACh release at the rat NMJ (344, 676). BoNT/A was reported to decrease arachidonic acid release from nerve terminal membrane stores, and it was suggested that this arachidonic acid deprivation would affect neuroexocytosis (498). The fact that the L chain of CNT possesses additional biological activities cannot be excluded, particularly in the light of the fact that the size of

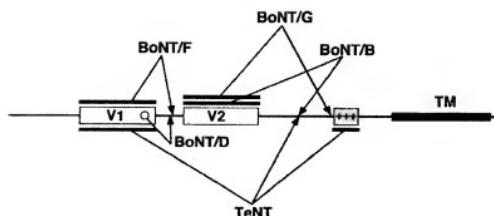


FIG. 7. Multiple interactions are involved in selectivity of L chain of clostridial neurotoxins for VAMP. These metalloproteases are highly specific for the 3 SNARE proteins, and their recognition is based on multiple interactions. Schematically shown is that VAMP-specific neurotoxins bind their substrate via either the V1 or the V2 copy of the SNARE motif while fitting the segment of VAMP to be cleaved within their extended zinc-containing active site (arrow). Tetanus neurotoxin also binds VAMP via a positively charged region located COOH-terminal with respect to the cleavage site. TM, transmembrane domain.

the L chains of CNT well exceeds that of most metalloproteases and should be carefully evaluated in future studies.

L. Clostridial Neurotoxins in Cell Biology

The peptide bonds hydrolyzed by each neurotoxin have been identified (Table 1). Apart from TeNT and BoNT/B, each one of the different CNT catalyzes the hydrolysis of a different peptide bond. Thus CNT are defined tools to probe the role of their targets in different cellular processes, and finer dissections of SNARE activities can be performed based on the different peptide bonds hydrolyzed by the different CNT. Moreover, because the three SNAREs are not cleavable by the CNT when they complexed (252, 465), these neurotoxins can be used to assay the state of SNARE assembly.

BoNT/A removes only nine residues from the SNAP-25 COOH terminus, yet this is sufficient to impair neuroexocytosis, thus indicating that this part of the molecule plays a relevant role in the function of the exocytosis apparatus. The fact that neuroexocytosis can be rescued in the NMJ poisoned with BoNT/A by LTX and by Ca^{2+} (see sect. III B), whereas BoNT/E poisoning cannot, indicates that the SNAP-25 segment comprised between the BoNT/A and /E cleavage sites (16 residues) is involved in a late stage of exocytosis taking place after ATP priming and conferring the Ca^{2+} dependence to the complex (29, 336, 664). Recently, this intermediate part of SNAP-25 was shown to determine the rapid removal of BoNT/E-cleaved SNAP-25, but not of BoNT/A-cleaved SNAP-25 in frog and human skeletal NMJ (164, 492).

It is presently very difficult to maintain in culture peripheral motoneuron, and this has hampered research on the morphological and functional consequences of CNT intoxication. Dissociated spinal cord neurons in culture, including primary motoneurons, are very sensitive to CNT (46, 234, 654). In addition, cortical brain neurons and granular cerebellar neurons are also sensitive to these neurotoxins (339, 450). In these cells, BoNT/A was found to cause no detectable morphological changes, whereas BoNT/C, which cleaves both SNAP-25 and syntaxin, causes rapid swelling of synaptic terminals followed by degeneration of axons and dendrites (657) and the collapse of growth cones in chick dorsal root ganglia (277). Electron microscopy revealed accumulation of vesicles at synapse, with little alteration of the soma cytosol. As a consequence of these cellular alterations, which develop from the nerve terminals, BoNT/C, uniquely among CNT, causes death of spinal cord neurons in culture (657), but not of other CNS neurons *in vitro* (450) (M. Leist, E. Fava, C. Montecucco, and P. Nicotera, unpublished observations), nor BoNT/C causes loss of motoneurons in humans (165) (R. Eleopra, V. Tugnoli, O. Rossetto, D. De

Grandis, and C. Montecucco, unpublished observations). Taken together, these results demonstrate the central role played by syntaxin in the control of the integrity of synaptic contacts, in addition to its essential function in exocytosis.

Cleavage of VAMP and of syntaxin by CNT leads to the release in the cytosol of a large part of their cytosolic portions. On the basis of their respective proposed roles as vesicular and target membrane SNARE, vesicle docking should be impaired in CNT-intoxicated synapses. On the contrary, it appears that poisoned and electrically silent synapses show an increased number of docked vesicles, as judged from electron microscopy (271, 388, 434, 450). Thus these results suggest that VAMP and syntaxin play additional role(s) in exocytosis and are possibly involved in vesicle reuptake as well.

Given the general role of SNARE in vesicular trafficking, the use of CNT is not limited to neuronal cells possessing CNT receptors. In the case of nerve cells or synaptosomes, the simple incubation of cells with CNT is sufficient to cause inhibition of neurotransmitter release and SNARE cleavage (9, 19, 67, 68, 250, 277, 339, 375, 378, 380, 382, 433, 450, 492, 525, 559, 657, 665, 666). In contrast, nonneuronal cells have to be permeabilized or microinjected (8, 10, 29, 53, 57–59, 77, 79, 136, 137, 196, 197, 265, 278, 307, 377, 405, 454, 468, 499, 500, 520, 584, 586). Incubation with very high doses of CNT may be sufficient to elicit effects with cells characterized by a large fluid-phase endocytosis (475, 626). Alternatively, cells can be transfected with the gene encoding for the light chain (6, 161, 332, 592).

The fact that the SNARE protein isoforms are involved in a variety of intracellular vesicle fusion events (216, 251), in addition to neuroexocytosis, has extended their potential range of use, but a word of caution is called for because more than one SNARE isoform can be inactivated by a given toxin within the same cell. It is not straightforward to predict the cleavability of a given SNARE by a given CNT from its sequence. In general, the cleavage site and one SNARE motif have to be preserved to be a toxin metalloprotease substrate, but the pattern of sensitivity of SNAP-25 and SNAP-23 from different species to BoNT/A and /E indicates that more information is needed to clarify this point (281, 353, 365, 411, 519, 631, 633). A useful development would be the design of novel metalloproteases of defined specificity based on CNT.

M. Regeneration of the Neuromuscular Junction Paralyzed by Botulinum Neurotoxins

Many bacterial and plant toxins cause cell death. The death of the poisoned animal follows a more or less extensive tissue necrosis (for references, see Ref. 495). None of the CNT is known to kill intoxicated neurons in

vivo, whereas they are extremely toxic to the animal because of the unique role of synaptic transmission in animal physiology and behavior. If a small amount of BoNT, dissolved in a minimal volume of carrier solution, is injected in the muscle, then the toxin does not spread around significantly. NMJ around the site of injection become paralyzed and lose their functionality, but the motoneuron and the innervated muscle fiber remain alive. However, the muscle undergoes a transient atrophy with loss of acetylcholinesterase staining and dispersion of ACh receptors from the end plate (14, 28, 157). Muscle fibers undergo a progressive atrophy with reduction of their mean diameter, which begins in the first 2 wk after BoNT injection and progresses for 4–6 wk. Differently from what happens when denervation is obtained by other means such as nerve ligation, anatomical contacts between the nerve and muscle are maintained in BoNT-treated animals, and there is no apparent loss of motor axons. For these reasons, BoNT are increasingly used to study NMJ plasticity. During BoNT-induced synapse remodeling, there is a very large increase of calcitonin gene-related peptide (CGRP), which can only partially be accounted for by the accumulation of CGRP-containing vesicles at the terminal (248, 523). In fact, such remarkable CGRP accumulation results also from an upregulation of its synthesis, caused by the release of trophic factors from paralyzed muscle fibers (248, 523). Under the effect of such growth factors, the motor end plate enlarges, and sprouts develop from the end plate itself, the terminal part of the axon and the nodes of Ranvier, and are guided to grow into the muscle fiber. Nerve terminal sprouts contain proteins involved in neuroexocytosis such as synaptophysin and synaptotagmin type II, which is the predominant NMJ synaptotagmin isoform (288). The number of motor end plates on a single muscle fiber also increases. Axon collaterals develop and lead to an increase in the number of fibers innervated by a single motor axon. Moreover, it is possible to identify some muscle fibers that are innervated by more than one motor axon. The structural alterations seen in BoNT-treated muscles parallel those documented in other forms of denervation. After axonal sprouting and reformation of functional nerve-muscle junctions, the muscle eventually regains its normal size and both acetylcholinesterase and ACh receptors reconcentrate at the NMJ. Later, sprouts largely degenerate and the end plate regains its normal morphology and function. The muscle atrophy induced by BoNT in animal models and in humans is therefore largely reversible, even after repeated BoNT injections (reviewed in Refs. 75, 598).

Nearly all these studies have concentrated on BoNT/A and have not tried a correlation with the BoNT/A-induced cleavage of SNAP-25. Recently, Raciborska et al. (492) have shown that cleavage of a small fraction of syntaxin and SNAP-25 at the frog NMJ is sufficient to

completely block ACh release. This demonstrates that pools of SNARE proteins exist at the in vivo toxin site of action, as previously shown for neurons in culture (see sect. *viJ*). It appears that a low proportion of the total SNARE present at the synapse is the one actively involved in neuroexocytosis and that a larger pool acts as a reservoir. Moreover, BoNT/E-truncated SNAP-25 was shown to be removed from the NMJ, whereas BoNT/A-truncated SNAP-25 remained in place. This finding is to be correlated with the fact that in humans the effect of BoNT/A lasts for months, whereas the effect of BoNT/E is reversed within a few weeks (164), as noticed before for the rat NMJ (409). Because this effect is not due to a different lifetime of the two neurotoxins inside the NMJ, this result was interpreted as an indication that the 25-residues-less SNAP-25 is nonfunctional in neuroexocytosis as well as in a system which monitors synaptic integrity. Therefore, it is rapidly replaced by newly synthesized SNAP-25 molecules with rapid recovery of nerve-muscle coupling. In contrast, the 9-residues-less truncated SNAP-25 generated by BoNT/A is nonfunctional in neuroexocytosis, whereas it somehow preserves other synaptic (structural) functions and is therefore only slowly replaced by new molecules (164).

N. Therapeutic Uses

The demonstration that the inhibition of the nerve-muscle impulse is followed by a functional recovery of the NMJ provides the scientific basis of the rapidly growing use of BoNT in the therapy of a variety of human diseases caused by hyperfunction of cholinergic terminals (285, 551). Injections of minute amounts of BoNT into the muscle(s) to be paralyzed led to a depression of the symptoms lasting a few months. BoNT/A is generally used, but other BoNT types are currently under clinical trial and, recently, very encouraging results have been obtained with BoNT/C (165). Those BoNT whose effect is not reversed by α -LTX or by increasing intrasynaptic Ca^{2+} are effective, but their beneficial effects have a much shorter duration (354) (Eleopra et al., unpublished observations). Injection of BoNT is currently recognized as the best available treatment for dystonias and for certain types of strabismus, and it is now being extended to several other human pathologies (285, 423). This treatment can be repeated several times, without major side effects such as the development of an immune response. If antineurotoxin antibodies are produced, treatment can be continued with another BoNT serotype.

TeNT is used to induce experimental epilepsy (25, 78) and neuronal degeneration (26) in animal models. More recently, the COOH-terminal one-third of TeNT, which retains most, but not all, of the neuronal binding and uptake properties of the entire toxin, has been used

as a carrier of lysosomal hydrolase (146), superoxide dismutase inside cells in culture (182, 191), or β -galactosidase in mouse embryos (115). These studies open the possibility of using TeNT as carrier of various biologicals from selected peripheral sites of injections to selected areas of the CNS.

O. Role of the Neurotoxins in Clostridial Ecology

This issue cannot be adequately dealt with at the present time because of the poor knowledge of the ecology of *Clostridia* in general and, in particular, of that of the toxicogenic *Clostridia*. A successful bacterium is the one able to multiply effectively and to spread in such ways that it is present in nature in large numbers (401). During evolution, such bacterium attains a state of balanced pathogenicity that causes the smallest alteration to host physiology compatible with the need to enter and multiply in the host body and to spread to other individuals. As noted above, genes coding for CNT are episomic. It was suggested that chromosomal genes code for proteins essential for the bacterial life cycle, whereas episomal genes are important for related functions, such as growth and spreading under unusual environmental conditions. Living vertebrates offer within their bodies only very small anaerobic habitats, where *Clostridia* can survive. The release of a neurotoxin kills the animal and converts it into an anaerobic fermentor able to support the growth of billions and billions of *Clostridia* of endogenous as well as exogenous origin. *Clostridia* are known to produce a variety of hydrolases that facilitate dissolution of tissues. In this simplified view, the production of CNT is functional to multiplication. During the massive *Clostridia* growth that takes place on cadavers, neurotoxin genes can be exchanged via bacteriophages or conjugational plasmids or transposons. Of course, the cadaver cannot support bacterial spreading to other hosts. Thus, when nutrients are consumed, *Clostridia* sporulate, and their spores are dispersed in the environment by atmospheric agents and other natural forces. On the basis of these considerations, toxicogenic *Clostridia* do not appear to be balanced pathogens. However, if one takes into account their anaerobic nature, killing the host is functional to the life cycle of a strictly anaerobic organism, and the production of spores is essential to their survival and spreading in the environment.

The finding that CNT are zinc-endopeptidases specific for different proteins of the neuroexocytosis apparatus, which are cleaved at different peptide bonds, suggests a possible evolutionary origin of the CNT. *Clostridia* produce a variety of rather nonspecific metalloproteinases that act outside cells (247). At a certain stage of evolution, a metalloproteinase gene fused with another gene giving rise to a protein able to act specifi-

cally at the NMJ. Further genetic rearrangements have then led to neurotoxins able to enter neurons and to selectively direct their proteolytic activity to proteins of the neuroexocytosis apparatus. Different sites of attack of the same supramolecular structure ensure that an animal species cannot become resistant to all CNT at the same time by point mutation of the site of proteolysis, as rats and chickens have done for TeNT and BoNT/B (459).

IV. NEUROTOXINS WITH PHOSPHOLIPASE A₂ ACTIVITY

A. Distribution and Toxicity

At variance from bacteria, animals produce venoms that are highly complex mixtures of toxins differing both in terms of targets and mechanism of action. The venoms of even very small animals, such as the marine predatory snails of the genus *Conus* or scorpions, are goldmines of pharmacologically active compounds, with several dozens of different toxins present in the same venom gland (448, 495). The richness and complexity of animal venoms was appreciated long ago but could be exploited only recently due to the development of refined separation techniques. Hundreds of different toxins are now available in pure forms (246, 257, 297, 495) and, surely, many more will become available as additional venomous species are investigated and after further developments in analytical biochemistry and molecular biology.

Major components of snake and insect venoms are disulfide-rich small toxins that exhibit phospholipase A₂ (PLA₂) activity; they hydrolyze the *sn-2* ester bond of 1,2-diacyl-3-*sn*-phosphoglycerides (297). More than 800 PLA₂ have been characterized so far [for reviews, see Dennis (141, 142) and Tischfield (601)]. PLA₂ of high molecular weight are at work inside cells in a variety of processes from phospholipid turnover to the production of inflammatory mediators. Ca²⁺-dependent PLA₂ of smaller size are secreted outside cells, and they include digestive and inflammatory enzymes, as well as toxins from snakes, lizards, and insects. Secreted PLA₂ are stabilized by many disulfide bridges (6 or 7), making them highly resistant to the proteolytic and denaturing conditions in which they have to operate. On the basis of their sequences, PLA₂ have been divided into various classes. However, despite amino acid sequence differences, PLA₂ fold into very similar tertiary structures. Relatively few variations appear to be sufficient to convert a nontoxic pancreatic PLA₂ into a highly toxic protein (98, 552). Similarly, few variations appear to be sufficient to address snake PLA₂ to different targets within the prey ranging from neurons to muscle cells, from red blood cells to platelets (297, 298). A variety of PLA₂ may be present within the same venom to increase its efficacy and rapidity of action on the prey.

Given the scope of this review, we deal only with presynaptic PLA₂ neurotoxins, whose major effect is a persistent blockade of ACh release at the NMJ causing animal death by respiratory failure. Some PLA₂ neurotoxins are not strictly specific for cholinergic terminals, as shown by the fact that when injected in the brain they exert a variety of effects due to inhibition of neurotransmitter release at a variety of CNS synapses, and some PLA₂ neurotoxins are also myotoxic (reviewed in Refs. 215, 242).

Most presynaptic PLA₂ neurotoxins have been isolated from snake venoms. Toxicity varies among species, and it would be useful to have toxicity data on the usual prey of each snake, because neurotoxic PLA₂ appear to have been tuned during evolution to increase their specific potency (242, 269). Sufficient comparative data are presently available only for mice, where their LD₅₀ toxicity values range from the 1 mg/kg taipoxin (isolated from the venom of the Australian snake *Oxyuranus scutellatus*) to the 750 mg/kg pseudexin B (isolated from the venom of *Pseudechis porphyriacus*). Rosenberg (508) has made an extensive analysis of the enzymatic activity and toxicity of PLA₂ and has concluded that, although data obtained in different laboratories are not always comparable, a strict correlation between their PLA₂ phospholipase activity and their toxicity is not apparent. It is clear that other factors, in addition to PLA₂ activity, such as diffusion in the body and neurospecific binding, come into play to determine the toxicity of these neurotoxins. Even taipoxin, the most potent of them, is several thousand times less toxic than CNT, and it is not appropriate to ascribe such a difference solely to their enzymatic activities. In fact, CNT and PLA₂ neurotoxins have different pharmacokinetic properties and distinct presynaptic receptors.

B. Structure and Enzymic Properties

More than 50 presynaptic neurotoxins endowed with PLA₂ activity have been characterized so far. These neurotoxins come in a complex array of forms with variations at all levels of protein structure. Sometimes, more than one PLA₂ neurotoxin and/or several isoforms of the same PLA₂ are secreted in the same venom (72, 187, 222). On the basis of their quaternary structures, they can be divided into four classes.

Class I comprises single-chain toxins of molecular mass varying in the range of 13–15 kDa with seven disulfide bridges. This class includes, among others, agkistrodotoxin from *Agiastrodon* snakes, ammodytoxin from *Vipera ammodytes ammodytes*, caudotoxin from *Bitis caudalis*, notexin from *Notechis scutatus scutatus*, OS toxin from *Oxyuranus scutellatus scutellatus*, and pseudexin from *Pseudechis porphyriacus*.

Class II includes neurotoxic PLA₂ composed of two noncovalently linked homologous subunits, at least one of which retains PLA₂ activity. Crototoxin and related rattlesnake venom neurotoxins (24 kDa) from snakes of the genus *Crotalus* are heterodimers of a basic enzymic subunit (2 isoforms of 12 kDa) and an acidic subunit (4 isoforms of 12 kDa) of no known biological activity (176). Similar dimeric neurotoxins are produced by several other snakes (72).

Class III includes heterodimers composed of unrelated subunits, such as the most studied of PLA₂ neurotoxin β -bungarotoxin. This toxin is manufactured by *Bungarus multicinctus* together with the ACh receptor specific α -bungarotoxin (310). It is composed of a 120-residue-long PLA₂ subunit disulfide linked to a 7-kDa protein, highly similar to dendrotoxin, a neurotoxin which binds specifically to voltage-gated potassium channels of presynaptic terminals and, accordingly, the two neurotoxins compete for presynaptic membrane binding (1, 13, 41, 64, 66, 153, 244, 324, 463, 478, 501, 502). In turn, dendrotoxin has a sequence and a structure very similar to that of the Kunitz-type trypsin inhibitor and to the three-foil subdomain of the receptor binding domain of Tent. This provides a remarkable example of evolution-driven reshaping of the same scaffold to radically change its binding specificity (324, 593, 611). Different β -bungarotoxins have been isolated so far with a similar PLA₂ subunit and different binding subunits (113). Their toxicity ranges from 19 to 130 mg/kg and does not appear to correlate with the level of PLA₂ activity (311, 508).

Class IV comprises noncovalently associated oligomers of homologous subunits. This class includes taipoxin, which is made of a strongly basic PLA₂ subunit of 120 residues, of a 120-residue-long nontoxic subunit, and of a 135-residue-long glycoprotein subunit with 8 disulfide bridges, which is nontoxic, but retains PLA₂ activity. Similarly, paradoxin from *Parademanisia microlepidotus* is also a heterotrimer. Textilotoxin is the most complex of these neurotoxins. It is produced by *Pseudonaja textilis textilis*, and it is a 70-kDa pentamer of homologous subunits, all of them having PLA₂ activity, two of which are disulfide bridged. The PLA₂ activity of textilotoxins is lower than that of its isolated subunit A, but the animal toxicity of the pentameric toxin is 1,000-fold higher (461). The high-resolution structures of three presynaptic PLA₂ neurotoxins, two notexins and one β -bungarotoxin, as well as those of 12 nontoxic PLA₂, have been determined (16, 98, 324, 552, 647). The three neurotoxin PLA₂ chains fold very similarly to pancreatic phospholipases with their characteristic six conserved disulfide bonds that greatly contribute to the stability and compactness of the molecule. The structure of notexin, shown in Figure 8, reveals the characteristic three α -helices with two β -strands. The protein is also stabilized and activated, by Ca²⁺ binding. The Ca²⁺ binding site

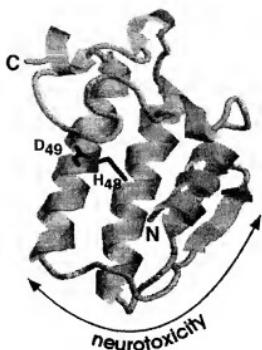


FIG. 8. Ribbon drawing of the 3-dimensional structure of notoxin. Position of the His-48 and Asp-49 residues essential for phospholipase A_2 activity is shown. Chemical modification experiments indicate that neurotoxicity is associated with the bottom part and righthand side of the molecule. This picture was obtained by using protein coordinates available in the PDB Databank with the accession number 1ae7. (Photo courtesy of Dr. B. Westerlund, University of Uppsala, Uppsala, Sweden.)

consists of the consensus sequence Tyr-Gly-Cys-Tyr/Phe-Cys-Xaa-Gly-Gly. Several divalent ions, including Sr^{2+} , Ba^{2+} , and Zn^{2+} , can bind to the same site but cannot substitute for Ca^{2+} in catalysis, and indeed, they are useful inhibitors of the PLA_2 activity. A remarkable feature of these enzymes is a hydrophobic channel that accommodates the fatty acid chains of the phospholipid molecule and places the ester bond to be cleaved into the active site. The key residues directly involved in catalysis are His-48, which hydrogen bonds the water molecule used for hydrolysis, and Asp-49, which positions the Ca^{2+} coordinating both the phosphate and the *sn*-2 carbonyl groups. The key role of this histidine residue is demonstrated by the total loss of enzymic activity caused by its chemical modification with *p*-bromophenacyl (669, 670). The Ca^{2+} atom plays a double role in catalysis; it contributes to the correct positioning of the substrate molecule, and it polarizes the ester bond, thus promoting the entry of the water molecule. The activity of these PLA_2 on phospholipid molecules inserted in a biological membrane or in micelles is much higher than that exerted on monomeric phospholipid molecules. This is due to the higher efficiency of interfacial catalysis, which depends on the absorption of the enzyme onto the lipid-water interface, strongly promoted by the presence of anionic amphipatic molecules within the membrane. Such interfacial membrane absorption promotes the diffusion of the phospholipid molecule from the membrane to the active site channel.

Chemical modification studies have indicated that segment 59–89, which includes a short α -helix (residues 58–66) and the β -pleated loop (residues 74–85) on the righthand side of the notoxin structure of Figure 8, is directly involved in determining neurotoxicity (thoroughly reviewed in Ref. 669). In the case of the heterodimeric β -bungarotoxin molecule, in addition to a single disulfide bridge, electrostatic, and hydrophobic forces, restricted within a long and narrow region, are involved in the protein-protein interaction among the two subunits (324). The K^+ channel binding region has been identified by comparison with other K^+ channel binding proteins as a basic elongated surface, at the opposite end of the antiprotease loop. Its relative orientation with respect to the enzymic subunit is such that, upon binding to the K^+ channel, it brings the PLA_2 active site onto the membrane surface (324). The limited subunit-subunit interaction explains why reduction leads to their separation with total loss of neurotoxicity (105), as is the case for the CNT (144, 537).

C. Presynaptic Activity of PLA_2 Neurotoxins

After the isolation of PLA_2 neurotoxins in pure form, their activity could be studied both *in vivo* and *in vitro* on isolated nerve-muscle preparations (187, 243). There is a large variability in the sensitivity of different animal species to the different PLA_2 neurotoxins (508). It is hence difficult to draw a precise picture of the symptoms that follow intoxication with these neurotoxins (80, 103, 104, 243, 293). In general, systemic acute intoxication after intraperitoneal or intravenous injections leads to death by respiratory failure due to paralysis of respiratory muscles. Such final events may be preceded by hyperexcitability (intoxication with β -bungarotoxins) or by a flaccid paralysis (intoxication with crotinin). Contrary to what was found with latrotoxins (see sect. v) or with curaremimetic neurotoxins, no matter how high the dose, there is always a minimum interval of ~1 h between injection of the PLA_2 neurotoxins and death (103, 243, 293). Presynaptic inhibition can be more conveniently studied *in vitro* with NMJ preparations, which provide a more homogeneous picture of the mode of action of PLA_2 neurotoxins. After the initial demonstration that β -bungarotoxin inhibits ACh release (103), many studies have been performed on these isolated preparations, and they have been discussed previously in several excellent reviews to which the reader is referred to for details and references (187, 242, 243, 269). Here, we simply summarize the main general conclusions. 1) PLA_2 neurotoxins strongly decrease the size of EPP and the frequency of spontaneous MEPP, without affecting their size. 2) A lag phase is always present between toxin addition to the bath and blockade of ACh release; the minimal duration of this lag phase is largely independent from the dose. 3) The time to the onset of muscle paralysis is reduced by nerve stimulation and in-

creasing temperature. 4) Following the amount of evoked ACh release as a function of time after toxin application, three subsequent phases can be distinguished at the NMJ poisoned by PLA₂ neurotoxins, although there may be large variation between animal species. As shown in Figure 9, a short initial phase displaying either decreased or unchanged ACh release is followed by a longer phase (10–30 min) of stimulation of evoked release, which then fades into the third phase (30–120 min) of complete and irreversible inhibition. 5) Some neurotoxic PLA₂ also inhibit voltage-controlled K⁺ channels at synaptic terminals. 6) Application of antitoxin antibodies and washing with fresh medium are effective in preventing the toxic effects only if performed within a few minutes after toxin addition. 7) At late stages, several alterations of the permeability properties of the plasma membrane and of synaptic organelles including mitochondria become apparent.

Ultrastructural analysis has focused mainly on the last phase of inhibition and shows a picture radically different from those of NMJ poisoned with CNT (111, 127, 214, 330). As can be seen from Figure 10, the main features are 1) swollen and enlarged axon terminals with an almost complete depletion of SSV, 2) appearance of several clathrin-coated W-shaped plasma membrane invaginations also in areas not facing the muscle, and 3) at later stages swollen mitochondria and vacuoles. Comparisons between the number of quanta released by the NMJ and the number of SSV present within the bouton have not been carried out, but the kinetics and morphological changes induced by the PLA₂ neurotoxins at NMJ suggest that they may both promote fusion of SSV with the presynaptic membrane, inhibiting at the same time SSV retrieval. Endocytosis appears to be blocked at a stage after formation of the clathrin scaffold, but preceding the clo-

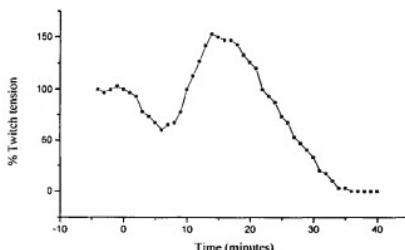


FIG. 9. The 3 phases of intoxication of the neuromuscular junction by taipoxin. A mouse phrenic nerve hemidiaphragm preparation was treated with taipoxin (3 µg/ml) at 37°C. Muscle twitch tension was recorded and is expressed here as percentage of the muscle tension value determined before toxic treatment. This experiment was performed by Dr. R. Dixon at the University of Padova.



FIG. 10. Ultrastructure of a motoneuron nerve terminal poisoned by notexin. A mouse motoneuron terminal 2 h after the injection of notexin in the muscle is shown. Notice the almost total depletion of small synaptic vesicles and the presence of numerous W-shaped structures also in the nerve terminal region not facing the muscle (arrows). [Photo courtesy of Dr. R. Dixon, as taken in the laboratory of Prof. J. B. Harris (University of Newcastle).]

sure of the vesicle, which requires dynamin, amphiphysin, and adaptor proteins (125).

Several studies have attempted to correlate the phospholipase activity of these neurotoxins with their toxicity *in vivo* and *in vitro* on various NMJ preparations. These experiments were analyzed in details in recent reviews (for references, see Refs. 187, 508), and therefore, only general conclusions are reported here. There appears to be little, if any, relation between the PLA₂ activity and the initial phase of inhibition of ACh release, but there is a partial correlation between PLA₂ activity and final inhibition of neurotransmitter releases, i.e., the turnover rate of the PLA₂ activity of these neurotoxins and the amount of hydrolyzed phospholipids are not sufficient to account for their toxicity and for the intensity of their effects at synaptic terminals (Fig. 9).

As clearly discussed previously (298), these neurotoxins possess a pharmacological site, which is responsible for their tissue and cell specificity and directs the PLA₂ enzymatic activity toward a subsite(s) of the presynapse of critical importance for neurotransmitter release. Kini and Evans (298) also proposed that the pharmacological site binds specifically to a protein or a glycoprotein of the presynaptic membrane rather than to a lipidic component. Enzymatic activity may not necessarily and always be needed for the display of the toxic activity, since presynaptic alteration may follow the binding and modification of the activity of a target protein.

In most cases, however, some PLA₂ activity is involved in the production of fatty acids and lysophospho-

lipids. Fatty acids are capable of inducing fusion of lipid bilayers (327, 381, 658, 674) and, although lysophospholipids counteract their fusogenic activity, *in vitro* vesicles were shown to fuse upon incubation with PLA₂ (290, 444, 515). These studies might be relevant to the *in vivo* mechanism of action of PLA₂ neurotoxins if one assumes that they are enzymatically active in the cytosol, like the CNT are. It should be noted, however, that the cytosolic Ca²⁺ concentration is at least four orders of magnitude lower than that required for PLA₂ activity (141), and it is not clear how long these highly disulfide-bridged enzymes preserve their activity in the strongly reducing environment of the cytosol. Moreover, the products of phospholipid hydrolysis, lysophospholipids, and fatty acids have high diffusion coefficients within the two-dimensional solvent constituted by a biological membrane. Accordingly, fatty acids and lysophospholipids are expected to diffuse out of the site of production very rapidly. Simpson and co-workers (572, 573) have performed various experiments aimed at testing the possibility that the PLA₂ neurotoxins are endocytosed and have concluded that this is not the case. We are left with the possibility that these neurotoxins act on the external surface of the presynaptic membrane at their site of binding (298). For reasons unclear at the moment, PLA₂ hydrolysis products could remain strongly associated with the neurotoxin receptor and modify its physiological function in such a way as to promote neuroexocytosis. The irreversibility of the inhibition caused by PLA₂ neurotoxins is in keeping with the hydrolytic nature of their enzymatic activity. An alternative hypothesis is that presynaptic PLA₂ neurotoxins, in addition to releasing the fatty acid from the phospholipid molecule, also transfer the fatty acid to proteins, similarly to diphtheria toxin, which is both a NAD⁺ glycohydrolase and an ADP-ribosyltransferase (118). Acylation of selected residues of the PLA₂ neurotoxin receptor could lead to a permanent modification of its function. In this respect, it is noteworthy that several proteins involved in neuroexocytosis contain several cysteine residues within their transmembrane segments. The multiple ultrastructural alterations that have been documented by electron microscopy may have provided pictures of a final situation, determined by the accumulation of multiple and sequential lesions. Indeed, with time, fatty acids and lysophospholipid molecules are expected to be produced in considerable amounts by the PLA₂, and they can spread to several synaptic terminals organelles leading to functional and morphological alterations. Fatty acids are uncouplers of mitochondrial oxidative phosphorylation and may induce ion permeation through membranes. Such molecular lesions were evident in synaptosomes (436, 516), with a drop in synaptic ATP level, a biochemical lesion which in turn affects all those structures and functions dependent on phosphorylation. In such a scenario, binding becomes the key initial event of the intoxication

of synapse by PLA₂ neurotoxins. Identification and characterization of the receptor of these neurotoxins appear essential to the understanding of their mode of action and to the identification of novel proteins and events involved in neurotransmitter release.

D. Membrane Binding of PLA₂ Neurotoxins

This crucial step of the activity of PLA₂ neurotoxins has been extensively and thoroughly investigated with a variety of techniques and extensively analyzed in recent reviews, where one can find all relevant references (72, 222, 329). Therefore, here we restrict ourselves to conclusions of general interest and to a discussion of the more recent literature.

Because several PLA₂ neurotoxins are active on central synapses, as well as on peripheral ones, most binding studies have appropriately used brain-derived nerve membranes in the form of synaptosomes, microsomes, or synaptic vesicles. Direct PLA₂ neurotoxin binding and competition experiments, employing a variety of radioactively labeled neurotoxins, were performed. Cross-linkers, affinity labeling, and affinity chromatography with matrix-bound neurotoxins were also used in an effort to isolate toxin receptors. These studies have shown that a limited number of high-affinity binding sites exist along with unsaturable low-affinity binding sites. Although it is possible that negatively charged lipids abundant on the external face of the presynaptic membrane are involved in low-affinity interactions, specific proteins or glycoproteins of the presynaptic membrane are believed to contribute to the high-affinity sites. Competition experiments have indicated that distinct receptors are involved in binding different neurotoxins. Moreover, although binding of class I monomeric PLA₂ neurotoxins is reversible, that of the multimeric class IV neurotoxins is largely irreversible. A multiple binding mediated by the single subunits, even via low-affinity interactions, would account for such apparent irreversibility. β -Bungarotoxin was demonstrated to bind specifically to presynaptic voltage-dependent K⁺ channels via its smaller dendrotoxin-like subunit (1, 13, 41, 64, 66, 153, 244, 324, 463, 478, 501, 502). Such a binding brings the PLA₂ close to the membrane surface (324). Cross-linking experiments have implicated a set of ill-defined proteins in the range of 36–88 kDa, termed N-type receptors, in the binding of other PLA₂ neurotoxins (reviewed in Ref. 329). Affinity chromatography with matrix-bound taipoxin has led to the identification of two proteins of 47 and 49 kDa. The 47-kDa molecule has been identified as a neuronal pentraxin, a neuron-specific protein with sequence similarity to pentraxins (147, 545), which are proteins secreted by various cells during the inflammatory and immune responses. The 49-kDa protein turned out to be a Ca²⁺-binding protein, localized in the

lumen of the endoplasmic reticulum, therefore called "taipoxin and Ca^{2+} -binding protein of 49 kDa" (TCBP-49) (147, 148). Other investigations have led to the identification of a large 180-kDa protein (average 1,460 residues in different species), expressed in several tissues, which is analogous to the macrophage mannose receptor (283), and has been termed the M-type receptor (329). This receptor binds OS2 as well as several nonneurotoxic PLA₂ via the region around the Ca^{2+} binding site, which is well conserved among the low-molecular-weight PLA₂ (141, 254). The possible significance of these findings for the presynaptic activity of PLA₂ neurotoxins is not as immediate as that of β -bungarotoxin binding to presynaptic K⁺ channels. As suggested by the authors (147, 148, 545), it is possible that neuronal pentraxin binds taipoxin and mediates its internalization within the Ca^{2+} -rich endoplasmic reticulum, where the toxin would be retained via its binding to the TCBP-49. It remains to be explained how phospholipid hydrolysis in the endoplasmic reticulum located in the cellular body would lead to inhibition of ACh release at the nerve terminal, which is located far away from the neuronal soma. New insights are expected from the characterization of the N-type acceptors of PLA₂ neurotoxins, because it is clear that neurospecific binding of PLA₂ neurotoxin is an essential step in their inhibition of neurotransmitter release.

V. NEUROTOXINS PROMOTING NEUROEXOCYTOSIS

A. Distribution and Toxicity

A large number of neurotoxins that promote neurotransmitter release are known, and they cause symptoms correlated with such excitatory activity. The evolutionary advantage of excitatory over inhibitory neurotoxins is not evident considering the life-style of predators, and there are even cases of venomous animals such as the stonefishes (227) that do not use the excitatory toxin to capture the prey at all. Toxins causing a rapid prey immobilization without convulsions, which may lead the prey to escape and become inaccessible to the predator, appear better suited to the need. It is possible that excitatory neurotoxins, which invariably induce strong pain reactions, were originally devised to warn predators, much like the electric discharge of electric fishes. As a matter of fact, a pain reaction is very effective in inducing a long-lasting memory of the encounter with the venomous animal. It is then possible that, later during evolution, the gene, encoding for an excitatory neurotoxin specific for vertebrate synapses, has been duplicated and reshaped in such a way as to redirect its specificity to invertebrate preys of smaller size. This may well have been the case of the α -LTX, a "warning" type of spider toxin directed against verte-

Latrodectus mactans tredecimguttatus spider venom together with as many as five homologous insect specific latrotoxins (α -LIT, β -LIT, γ -LIT, δ -LIT, ϵ -LIT) and one crustacean specific latrotoxin (α -LCT) (219, 221). Hence, it appears that gene duplication starting with the ancestor *LIT* gene has taken place several times, perhaps with the advantage of developing insect specificities that are not apparent at the present time. In any case, convulsive reactions of an insect prey would not prevent accessibility to the highly mobile venomous spider because of its comparatively low size.

Whatever the role of excitatory toxins in the ecology of venomous animals, most of them alter the activity of ion channels and thus facilitate neuroexocytosis indirectly (257, 495). On the other hand, excitatory neurotoxins such as those contained in the venoms of the black widow type of spiders (genus *Latrodectus*) and of the stonefishes are known to directly induce neuroexocytosis by promoting a massive release of neurotransmitter. Envenomations by spiders of the genus *Latrodectus* cause lactrodectism, a poisoning syndrome that develops within an hour of being bitten, with pain first localized at regional lymph nodes. Rapidly, generalized muscle contractions and cramps develop together with hypertension and transient tachycardia followed by bradycardia. Abdominal muscle rigidity, profuse sweating, and oliguria are also associated in most cases (364, 431). Similarly, stonefish stings cause a pain sensation that starts from the site of injection and gradually diffuses and becomes stronger, reaching regional lymph nodes (227). General symptoms develop and are characterized by respiratory distress, transient hypertension followed by a prolonged hypotension, bradycardia, and muscle convulsions. In addition, the venom of these fishes has hemolytic and anticoagulant activities (227). A report of human stonefish envenomations described pulmonary edema with need of mechanical ventilation (338). All these symptoms can be ascribed to hyperexcitability of various nerve terminals, and this specificity is mirrored by sensitivity of different excitable cells in vitro (see sect. vC).

Application of black widow spider venom extracts to the frog sartorius NMJ induces a rapid and enormous increment of MEPP frequency that remains elevated for 10–15 min and then progressively declines to zero level, when no more SSV are present within nerve terminals (114, 351). Similarly, the *Synanceia trachynis* (stonefish) venom applied on frog and mouse nerve-muscle preparations triggers a rapid ACh release, followed by depletion of the synaptic terminals (317). Both venoms overcome the inhibition caused by BoNT/A (117, 202). Moreover, in most nerve terminal preparations, LTX is fully effective in the absence of Ca^{2+} , provided that millimolar concentrations of Mg^{2+} or other divalent cations are present in the bathing medium (218, 317, 404). Fesce et al. (179) estimated that, in the absence of Ca^{2+} , the *Latrodectus*

venom induces at the frog pectoral NMJ the release of >1,500 MEPP/s, rapidly declining to <10 MEPP/s and that during this period (<30 min) $\sim 7 \times 10^6$ MEPP were released, a value corresponding to the total estimated SSV contents of such nerve terminals. In contrast, in the presence of extracellular Ca^{2+} , ~400 MEPP/s were released in a sustained mode for longer than 1 h and a total of $>17 \times 10^6$ MEPP were released, convincingly showing that more than one turnover of SSV took place at the poisoned NMJ. Hence, it appears that LTX promotes SSV fusion with the presynaptic plasma membrane, but, in the presence of external Ca^{2+} , does not at the same time inhibit SSV recycling. Another venom, known to contain toxin(s) promoting extensive neurotransmitter release, is produced by the polychaete annelid worm *Glycera convoluta* (356, 362, 426). It contains a 300-kDa protein responsible for the effects, but this interesting neurotoxin has not been studied further.

The venom of *Latrodectus mactans tredecimguttatus* species of black or brown widow spiders has been studied biochemically in more detail than that of any other spider. However, it is likely that similar components are present in other spider species of the same genus because symptoms caused by envenomation are similar (364). Fractionation of this venom has led so far to the identification and purification of one LTX (193, 194, 217) and of five LIT and one LCT (158, 221, 308, 314). Trachynilysin, a protein toxin of ~150 kDa, has been isolated from the venom of the stonefish *Synanceia trachinis* (117), and similar-sized stonustoxin and verrucotoxin were purified from *Synanceia horrida* (209, 476) and from *Synanceia verrucosa* (203, 204), respectively. The effects caused at vertebrate nerve terminals by LTX are the same as those caused by the unfractionated venom, since LIT and LCT act specifically on invertebrate and crayfish synapses, respectively, and are ineffective on vertebrate synapses (221). The mouse LD₅₀ of purified LTX is 20 ng/kg (221), and that of the venom is 0.15 mg/kg (272). The LD₅₀ of LIT on *Galleria mellonella* larvae varies between 15 mg/kg for α -LIT and 1 mg/kg for ϵ -LIT, and the LD₅₀ of LCT on the crayfish *Procambarus clarkii* is 0.1 mg/kg (221). Because of their more recent characterization, much less is known about the stonefish presynaptic toxins. Available data indicate that the neuromuscular effects caused by the venoms are reproduced by the toxins (117, 227). The mouse LD₅₀ of stonustoxin is 17 mg/kg (476).

NMJ exposed to LTX have been studied thoroughly with various microscopic techniques, and these studies have been carefully reviewed previously (101, 476). The action of the toxin has been more recently visualized with the help of the styryl dye FM1-43, which is reversibly incorporated in the luminal face of the SSV membrane during exocytosis and is therefore used to monitor SSV recycling at nerve terminals (256). The morphological changes induced by a stonefish toxin have been described

only recently (117). The overall picture of different NMJ exposed to significant amounts of the excitatory neurotoxins for prolonged times is very similar to the one reported in Figure 11. It shows enlarged and swollen synaptic boutons, almost totally depleted of SSV, with preservation of most, if not all, dense-core vesicles. Mitochondria are also swollen with altered inner membrane morphology and accumulation of calcium phosphate precipitates. The massive SSV fusion with the presynaptic membrane triggered by these excitatory neurotoxins takes place at active zones (100, 491), in contrast to PLA₂ neurotoxins, which cause indiscriminate fusion all over the presynaptic membrane (127). Remarkably, dense-core granules are not induced to fuse by LTX or trachynilysin and remain inside the synapse (117, 371). The picture is substantially different depending on the presence or absence of Ca^{2+} in the bathing medium, the time of incubation

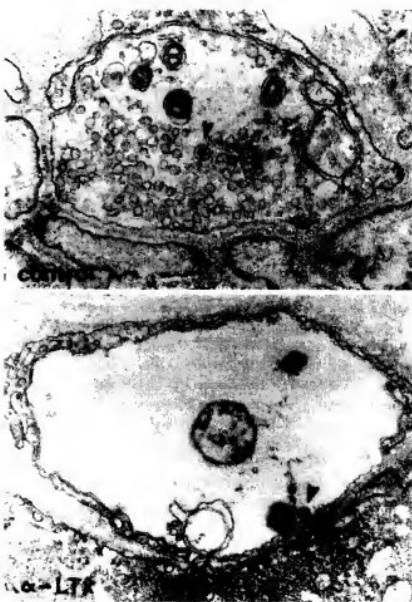


FIG. 11. Frog neuromuscular junction treated with α -latrotoxin (α -LTX). Exposure to high amounts of toxin for hours causes a massive release of small synaptic vesicles. This results in an enlargement of the plasmalemma and a total depletion of the neurotransmitter-containing vesicles, but not of the large dense-core vesicles containing neuropeptides (arrow). Nerve terminal is swollen as consequence of a toxin-mediated entry of cations. [From Matteoli et al. (371).]

tion, and the amount of toxin used. In the presence of Ca^{2+} , alterations are always less profound, with better preservation of residual normal SSV and mitochondria (101, 256, 617). FM1-43-stained SSV are present at the frog NMJ intoxicated with LTX in the presence of Ca^{2+} , indicating that endocytosis can still take place under these conditions (256). When Ca^{2+} is absent from the extracellular medium, the dye is not taken up at poisoned terminals, but staining resumes upon Ca^{2+} addition. Together with the electrophysiological experiments mentioned earlier (179), these data indicate that LTX promotes neuroexocytosis and does not inhibit synaptic vesicles recycling.

At variance from the CNT, all the described effects are triggered by the binding of these excitatory neurotoxins to the presynaptic membrane, not followed by their internalization. Indeed, injection of the antisera specific for the various venoms relieves pain and other symptoms even several hours after envenomation (227, 364).

B. Structure of Excitatory Neurotoxins

In general, neurotoxins that stimulate neuroexocytosis directly are large proteins, and the reason(s) for such complexity is not apparent. Structural information is limited to the primary sequences of part of them (158, 203, 209, 302, 303). Figure 12 compares schematically LTX, α -LIT, and δ -LIT. These neurotoxins are significantly similar, suggesting a common ancestral origin. They are made as inactive precursors that are trimmed at the NH_2 terminus, to remove a secretion signal sequence, and at the COOH terminus to produce the mature and active neurotoxin, as found in the venom gland. Thus the LTX, α -LIT, and δ -LIT consist of 1,381, 1,376, and 1,186 residues, respectively. Four different regions can be identified in the primary structure of these proteins. Part I is a poorly

conserved signal sequence, which is removed during toxin maturation, whereas part II is highly conserved and contains two putative membrane inserting segments, which are likely to be directly involved in the channel-forming properties of these neurotoxins in planar lipid bilayers (184, 273, 403). Part III is less conserved among the three latrotoxins and shows no similarity with known proteins, apart from the presence of many ankyrin repeats, a feature not shared by any other neurotoxin. Ankyrin repeats are present in a variety of proteins involved in very different functions and have been shown to mediate the binding of several plasma membrane proteins, including anion channels, to the cytoskeleton (45). A close inspection of these latrotoxin repeats reveals that only the NH_2 -terminal ones conform to the ankyrin motif, whereas the COOH-terminal repeats show large deviations and are not conserved among the different latrotoxins. Such differences would be in agreement with the possibility that the regions including the COOH-terminal ankyrin-type repeats (shaded in Fig. 12) are involved in presynaptic membrane binding (221). This region could be hidden by domain IV, which has variable size in the three latrotoxins and is not conserved; this feature could explain the lack of activity of the precursor latrotoxins. Indeed, part IV is removed during maturation and activation of the latrotoxins that takes place during venom maturation. δ -LIT found in the venom is trimmed at both NH_2 and COOH ends to 991 residues, and LTX is endoproteolytically cleaved after the 22nd ankyrin repeat (158, 221, 276). The reason(s) underlying such COOH-terminal proteolytic maturation and activation process is not known but may be related to efficient folding of the newly synthesized molecule and to stabilizing interactions with toxin-associated nontoxic low-molecular-weight proteins (205, 469). Several cysteines are present in the sequences of latrotoxins, but only three of them, located in domain

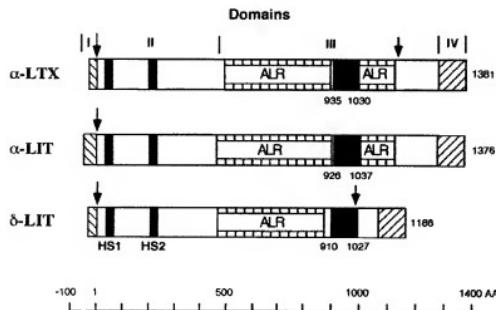


FIG. 12. Schematic structure of the latrotoxins (LTX). Primary structure of latrotoxins can be divided into 4 parts. An NH_2 -terminal signal sequence, which is removed (arrow) during toxin maturation, is followed by a conserved domain that includes 2 putative transmembrane segments (HS1 and HS2, black boxes), which are suspected to be involved in toxin ion channel formation. The third part is less conserved and contains several ankyrin-type repeats (ALR), particularly within a 100-residue-long segment (dark gray), which could be implicated in receptor-binding. COOH-terminal part IV is not conserved and is proteolytically removed (arrows) during maturation and activation of the precursor toxin molecule.

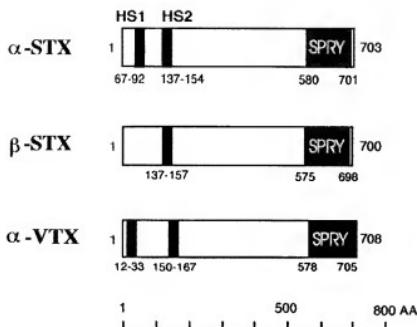


FIG. 13. Schematic structure of stonefish neurotoxins. These proteins consist of 2 noncovalently linked homologous subunits of ~700 residues (*a* and *b*). Similarly to the latrotoxins, the stonefish neurotoxins include within their NH₂ terminus 2 uncharged segments (HS1 and HS2), which are likely to be involved in forming the transmembrane sector of the toxin ion channel. These neurotoxins contain within their COOH termini a SPRY domain with internal repeats, whose function is not presently known. They are suggested here (see text) to play a role in binding as yet unidentified presynaptic receptors. STX, stonustoxin; VTX, verrucoxin.

II, are conserved. Disulfide bonding patterns have not been determined, but it is likely that they stabilize the molecule as they do in the PLA₂ neurotoxins. In addition, they play a fundamental role in activity, since reduction of LTX with 2-mercaptoethanol destroys its activity by preventing receptor binding (135). The unraveling of the structure-function relationship of latrotoxins will be greatly facilitated by the recent cloning and expression of active recombinant δ -LIT (158) and LTX (276). These studies have already shown that part IV is not required for activity and that each of the three conserved cysteines of LTX is essential for activity, since their single replacement led invariably to inactive mutants (276).

Comparatively, much less information is available on the stonefish neurotoxins. They are composed of two subunits of similar size, associated via noncovalent interactions (Fig. 13). The entire sequence of stonustoxin (209), of the β -subunit of verrucotoxin (203), and of 37 residues of the NH₂ terminal of the β -subunit of trachynilysin is presently available (117). The α -subunit (699 residues) and β -subunit (702 residues) of stonustoxin are highly homologous (50% identity), suggesting that their genes derive from duplication of a common ancestor gene (209). Ten cysteines of the heterodimer form disulfide bonds, to increase the toxin stability, while five additional cysteines have free sulfhydryls and are required for toxicity (295). Two uncharged segments located, similarly to the latrotoxins, within the NH₂-terminal region (black

boxes in Fig. 13), could account for the in vitro channel-forming and cytolytic action of stonustoxin (109, 209, 352). In addition, an analysis conducted with programs available from the EMBL Information Service shows that the stonefish neurotoxins contain several SPRY repeats within their COOH-terminal region. Such SPRY motifs have been previously identified in the ryanodine receptors, in the dual-specificity kinase SPIA from *Dicystostelium discoideum* (480) and in a family of proteins of unknown function (262). If it is assumed that the COOH-terminal region is implicated in receptor binding, as is the case for the CNT, then it is tempting to suggest that binding of presynaptic acceptors to ankyrin repeats and to the SPRY domain (both modules previously implicated in protein-protein interactions) could cause their clustering, thus affecting the induction of neurotransmitter release. Such a feature would contribute to account for the large difference found in the action of these neurotoxins on different animal species, cells, and synapses because not only the number but also the density of acceptors would be critical in determining neurotoxin binding and effect.

No information on glycerotoxin, which was only partially purified from *Glycera convoluta*, is available apart from the molecular mass, estimated to be ~300 kDa (362, 426). In none of these neurotoxins has the region responsible for activity been mapped.

C. Binding and Mechanism of Action

The rapidity of the stimulatory action exerted by these neurotoxins and the prolonged efficacy of the antiserum treatment point to a toxin activity displayed mainly at the level of the presynaptic membrane, with a minor, if any, contribution of intracellular toxin activities. Competition experiments indicate that LTX, trachynilysin, and glycerotoxin bind to different receptors within the presynaptic membrane (117, 356, 426), in agreement with their different amino acid sequences. Such binding has been thoroughly investigated only for LTX, taking advantage of its availability in pure form and of the preservation of toxicity after radioiodination (383). There is a close correlation between LTX binding and induced neurotransmitter release. Scatchard plot analyses have been necessarily made with synaptosomes or cells in culture and indicate the existence of high-affinity binding sites in the 0.1–10 nM range (383, 386). It is not, however, clear to what an extent these figures can be extrapolated to the NMJ *in vivo*. Two types of high-affinity LTX receptors have been distinguished in neuronal membrane on the basis of their Ca²⁺ dependence (208, 510). Monoclonal antibodies mapping of the surface of LTX revealed that additional regions of the molecule, different from the binding site,

are involved in its mechanism of action (99). Such possibility is suggested by the recent preparation of an LTX mutant that binds to brain membranes similarly to wild-type LTX but does not induce release of glutamate from synaptosomes (276). LTX receptors are selectively confined to the presynaptic membrane of the frog NMJ (618), but they are not restricted to release sites in the *Torpedo* electric organ (347) and are randomly distributed over the entire surface of isolated cells in culture (220, 521). Analysis of the distribution of these receptors in the brain shows a widespread distribution with a higher density in the hippocampus, the cerebral cortex, and the granular layer of the cerebellum (360). LTX not only induces the release of all neurotransmitters but is also capable of inducing the exocytosis of catecholamines from chromaffin cells and pheochromocytoma cells (32, 60, 218, 309, 349, 385, 397, 636), and of insulin from cells of pancreatic origin (334). Such a wide spectrum of cell targets indicates that the receptor(s) and the mechanism(s) triggered by LTX are present and conserved in a wide variety of cells endowed with exocytosis activity.

Binding of LTX to the plasmalemma of neuronal cells causes an influx of Ca^{2+} that has been documented both in intact cells and in synaptosomes with a variety of techniques. With a delay of a few dozens of seconds attributable to diffusion and binding of LTX to its receptor(s), long-lasting channels, which are nonselective for cations, lead to a large influx of Ca^{2+} and Na^+ with a consequent membrane depolarization (145, 218, 349, 384, 435, 509, 632). The conductance of LTX single channels, recorded by patch clamping of PC12 cells, was estimated to be 15 pS (632), but LTX channels in a neuroblastoma-glioma cell line have a much larger conductance (300 pS), and this difference may be related to the different amount and/or type of toxin receptors in the two cell lines. LTX channels in the latter cell line are permanently opened in Ca^{2+} -free solutions and are blocked by La^{3+} (273). The assembly of cellular ion channels does not depend on the presence of extracellular Ca^{2+} (384). It is presently unknown whether these channels are formed by the toxin itself, or by the toxin together with endogenous plasma membrane molecule(s), or if the toxin activates directly or indirectly an endogenous channel(s). However, the fact that purified LTX forms channels of large conductance (hundreds of picoSiemens) in planar lipid bilayers (184, 505, 529) and that toxin receptors greatly stimulate LTX channel formation (529) supports the first possibility.

The channels formed by LTX have been proposed to protrude on both sides of the membrane and to change conformation depending on membrane potential (106). Experiments performed on *Xenopus* oocytes and on lipid bilayers suggest that LTX channels are organized in clus-

ters, with openings cooperating into groups of bursts (183, 313). Moreover, LTX pores appear to be large enough to be capable of mediating a direct release of neurotransmitters from the cytosol, which is Ca^{2+} independent (134, 379). Also, α -LT and δ -LT form ion channels in planar lipid bilayers (158, 557), but they have much smaller conductances. Hence, the formation of toxin pores in the lipid bilayer of plasma membranes is a general property of latrotoxins, one which may explain a large part of the toxin-induced massive release of neurotransmitters.

Ion influxes through LTX channels are such that can well account for the massive, but time-limited, neurotransmitter release as well as for membrane depolarization and swelling of synaptic terminals. This fact does not exclude the possibility that LTX induces neurotransmitter release via binding to a receptor involved in the modulation of the secretory machinery that enhances the depolarization-evoked exocytosis (349).

As is the case with the spider venom, LTX is capable of inducing neurotransmitter release even in the absence of extracellular Ca^{2+} , provided that the medium is supplemented with millimolar concentrations of other divalent cations (2, 94, 95, 101, 404, 510). Contradictory results on its dependence by Ca^{2+} mobilized from intracellular stores have been reported with negative (2, 384) and positive correlations (134). In the absence of external Ca^{2+} , synaptic vesicle exocytosis is not followed by endocytosis, showing that extracellular Ca^{2+} are required for vesicle endocytosis (101). Unlike the Ca^{2+} -dependent LTX-induced neurotransmitter release, which is mediated via SNARE proteins and is blocked by CNT, the Ca^{2+} -independent release appears to be at least partially attributed to the LTX pores. However, other transmembrane signaling events are set in motion by LTX, which stimulates phosphoinositides hydrolysis upon binding to receptors located on the plasma membrane in PC12 cells (220, 627), as well as on synaptosomes (276). Whether phospholipase C stimulation plays a role in LTX-induced exocytosis is still controversial. Indeed, inhibitors of phospholipase C also inhibit the Ca^{2+} -dependent LTX-evoked exocytosis (134), but an LTX mutant with an insertion between the second and third domain binds normally and stimulates phosphatidylinositol hydrolysis without inducing neuroexocytosis from synaptosomes (276).

Taken together, these biochemical studies indicate that the action of LTX at nerve terminals is complex and results from the interaction with different receptors as well as from the direct formation of toxin channels. Multiple functional and structural changes are triggered in the presynaptic membrane by LTX binding and insertion in the lipid bilayer. Consequently, it is expected that the contribution of the different toxin activities varies at different synapses depending on the nature of the synapse, its level of exocytic activity, and the amount of toxin

present. In any case, unlike for the CNT, the key step in the mechanism of action of the latrotoxins and of the stonefish neurotoxins is their binding and insertion into the presynaptic membrane.

This knowledge has provided the impulse for various attempts to isolate LTX binding proteins, which have almost invariably employed detergent-solubilized brain membranes affinity chromatographed on matrix-bound LTX (133, 135, 314, 315, 340, 470, 472, 528, 613). These efforts have led to the characterization of neuroligin Ia as a Ca^{2+} -dependent LTX binding protein and to the discovery of the neuroligin family of proteins that exists in a multitude of isoforms in the mammalian brain (208, 406, 608). The binding of LTX to neuroligin Ia has been particularly well characterized, also with the help of neuroligin Ia knock-out mice and was shown to depend strongly on Ca^{2+} (133, 208, 471). However, neuroligin cannot be the sole functional receptor of LTX in vivo because its LTX binding is Ca^{2+} dependent and because glutamate neuroexocytosis can still be induced by LTX, although to a lower extent, from synaptosomes isolated from the brain of neuroligin Ia knock-out mice (208). It will be particularly interesting to determine the LD_{50} of LTX in these mice.

A Ca^{2+} -independent LTX binding molecule of 120 kDa has been recently identified (315, 340). It is a 1,471-

residue-long integral membrane protein with a large NH_2 -terminal extracellular part (871 residues), a seven-transmembrane segments membrane sector, and a cytosolic part (361 residues) (Fig. 14). There is disagreement on the actual size of the Ca^{2+} -independent LTX receptor; one group reported the value of 120-kDa protein (340) and another one presented evidence that the receptor is actually an heterodimer composed of the 120-kDa protein and of its 80-kDa fragment resulting from proteolytic removal of most of the extracellular portion (315). Several putative domains were identified in the Ca^{2+} -independent LTX receptor by homology searches. A NH_2 -terminal cysteine-rich part with homology to galactose-binding lectins is followed by a domain similar to olfactomedin, a protein of the extracellular matrix of the olfactory neuroepithelium. The second half of the extracellular domain is rich in cysteines and prolines and includes several sites of potential glycosylation. The membrane sector has strong similarities with the corresponding portion of the secretin receptor family, which also includes receptors for VIP, glucagon, calcitonin, and corticotropin-releasing factor. All these proteins are known to be involved in ligand-triggered secretion reactions mediated by large GTP-binding proteins (76, 325, 609). Remarkably, the 120-kDa LTX binding protein was indeed found to be associated with the G protein α -subunit (340).

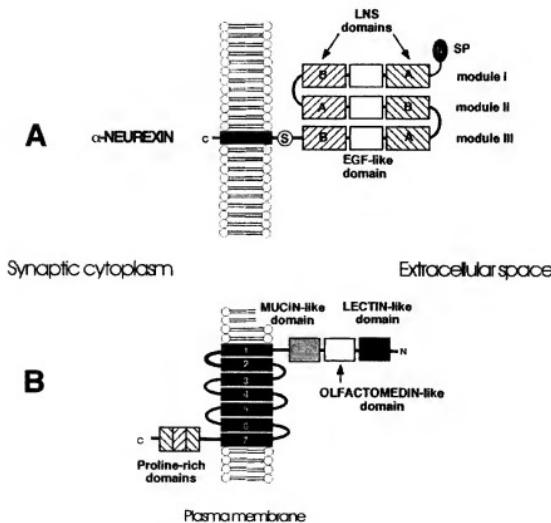


FIG. 14. Membrane topology and functional domains of latrotoxin receptors. *A:* schematic structure of the α -neurexins that bind LTX with high affinity in the presence of Ca^{2+} . The molecule begins with an NH_2 -terminal signal sequence (SP) followed by 3 large repeats, each one composed of 3 domains. The first and third domains are termed LNS because they are present in laminin, in neurexins, and in sex hormone binding proteins. The two LNS domains flank a central epidermal growth factor (EGF-like) domain. The 3 large repeats are connected to a single membrane-spanning segment via a sequence predicted to contain an O-linked carbohydrate binding site (S). The molecule ends with a short cytosolic tail. [Scheme adapted from Missler et al. (406).] *B:* Ca^{2+} -independent LTX receptor is predicted to contain 3 distinct domains in its extracellular portion. A sugar-binding portion is followed by a protein-binding domain, homologous to olfactomedin, and by a domain likely to be involved in binding components of the extracellular matrix (mucin-like domain). The integral membrane sector is composed of 7 membrane-spanning helices likely to interact with a trimeric G protein. It is followed by a COOH-terminal proline-rich cytosolic domain putatively involved in protein-protein interactions with unidentified cellular protein(s). [Scheme adapted from Krasnoperov et al. (315) and Lelianova et al. (340).]

Several pieces of evidence support the proposal that the 120-kDa protein is the bona fide Ca^{2+} -independent receptor of LTX (315, 340): *1)* this protein is only expressed in nervous tissue, *2)* transfected COS cells bind LTX with an affinity comparable to nerve cells, *3)* LTX effectively induces catecholamine release from chromaffin cells overexpressing the 120-kDa receptor protein, and *4)* insulin-secreting cells expressing neuromedin release insulin upon treatment with LTX only after transfection with the 120-kDa protein (334), although the lack of involvement of neuromedin in this specific case can be explained in several different ways.

It is particularly intriguing that this Ca^{2+} -independent LTX receptor copurifies with syntaxin, synaptotagmin, and the $G\alpha$ protein (340) because, recently, a neuronal Ca^{2+} channel was found to be regulated by a large G protein in a manner dependent on the presence of an intact syntaxin molecule, a regulation lost upon treatment with BoNT/C (580, 582, 660). Taken together, these findings suggest that the early phase of the LTX-induced Ca^{2+} current could be due to an endogenous Ca^{2+} channel, whose opening is induced by a conformational change of the receptor molecule, triggered by the binding of LTX. A short lag phase would characterize the toxin action because of the time necessary for binding and the time needed for the G protein-mediated receptor- Ca^{2+} channel coupling. The following, larger, Ca^{2+} current would be supported by a channel made of oligomeric LTX.

Together, these studies allow one to speculate on the possible structure and regulation of the large multicomponent apparatus that assembles at the active zone of the presynaptic membrane to carry out neuroexocytosis (48, 251, 346, 514, 590, 662), in the line of what done by O'Connor et al. (447). The presynaptic section of this apparatus could include a Ca^{2+} channel/syntaxin complex coupled to neuromedin Ia and the Ca^{2+} -independent LTX receptor via a $G\alpha$ protein. Neuromedin could be connected to active zones of neuroexocytosis via synaptotagmin interactions (472). A role in exocytosis for a large G protein was proposed long ago on the basis of indirect evidence obtained with mast cells (213), and it is in agreement with experiments performed with insulin-secreting cells (333). The role of the LTX receptor in the process could be that of providing a final control of the correct assembly of the neuroexocytosis machinery. Binding of LTX would cause a conformational change of its receptor(s) that would transmit a positive signal and directly induce neuroexocytosis. Such positive signal appears to be sensed also by the Ca^{2+} channel that opens, and in the presence of extracellular Ca^{2+} , amplifies the LTX neurotransmitter release via letting Ca^{2+} in. Binding to the toxin receptors is also instrumental in the subsequent LTX membrane insertion and assembly of LTX channels that would mediate larger ion fluxes, which in turn would

be responsible for the massive exocytosis, characteristic of this neurotoxin.

D. Use of α -LTX

Because of its specific properties, LTX has proven to be a very useful tool in neurobiology (360, 386). It has been used on both central and peripheral synapses of a variety of vertebrates showing no neurotransmitter specificity. LTX acts as well on isolated cells in culture, provided that they possess the appropriate receptor (for references, see Ref. 509). In particular, the use of LTX in combination with electrophysiology and electron microscopy techniques has provided a direct demonstration of the vesicular theory of neurotransmitter release. A correlation between changes in ultrastructure and toxin-induced discharge of MEPP showed that the total number of quanta discharged as a consequence of the toxin action corresponded quite well with the decrease in vesicle density. Therefore, each MEPP recorded from the postsynaptic cell is produced by the release of one quantum of neurotransmitter originally stored in the lumen of a single synaptic vesicle (101, 102, 274, 635). Work with neurotoxins has provided further support to this theory since five clostridial neurotoxins block neuroexocytosis via cleavage of VAMP, a protein which is characteristically localized on the synaptic vesicle-limiting membrane. More recently, LTX has been employed to define the unquenched nature of miniature events in CNS neurons (192).

LTX has also been widely used as a tool to study the membrane redistribution of vesicular protein antigens taking place during exocytosis with the aim of obtaining information on the mechanism of the process. Experiments carried out at the frog NMJ revealed that a specific molecular identity of the synaptic vesicle membrane is strictly conserved during the active exo-endocytic cycle. Indeed, no intermixing between components of the vesicle and the plasmalemma was found to take place when LTX was applied in the presence of extracellular Ca^{2+} , a situation in which the vesicle population is maintained by active recycling (617). On the other hand, a spreading of synaptic vesicle components in the plane of the plasma membrane takes place when the retrieval of synaptic vesicles is blocked (605, 617). With a similar experimental approach, it was shown that the synaptic vesicle-associated proteins synapsin I and rab 3a do not dissociate from the vesicle membrane before fusion and are translocated to the cell membrane together with the vesicle membrane (372, 605). Finally, the use of LTX and trachylysin at the frog NMJ has clearly indicated that the exocytosis of ACh-containing SSV and of peptide-containing large dense-core vesicles is regulated via different mechanisms (117, 371).

VI. CONCLUDING REMARKS AND FUTURE DEVELOPMENTS

The study of the mechanism of action of presynaptic neurotoxin has recently provided a wealth of novel information that has greatly changed and extended our knowledge of the process of neuroexocytosis. At the same time, this new knowledge has provided the molecular basis for further discoveries following their use as tools in neurobiology and cell biology (495). A remarkable development is the ever-growing utilization of BoNT in the therapy of human diseases, which was initially limited to dysfunctions of NMJ, but it is presently and successfully being extended to diseases due to hyperfunction of autonomic cholinergic terminals (285, 423). Also of great potential is the use of some Ca^{2+} channel inhibiting toxins from *Conus* snails and scorpions as blockers of the pain sensations at the spinal cord level. Novel uses can be envisaged for TeNT and other neurotoxins as carriers of biologically active molecules to particular areas of the cell or of the body.

Many new toxins will be discovered with future investigation of the venom of venomous animals and with the extension of research to other venomous animals. Moreover, it appears that toxicogenic bacteria are continuously evolving genes involved in their interaction with the vertebrate hosts (185), and novel bacterial toxins are likely to be characterized.

Another potentially rewarding line of investigation will be that of using the tools of molecular genetics to design new toxins endowed with pinpointed binding and enzymatic activities to be used as defined biochemical and therapeutic tools.

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Review of the literature is limited to December 1998. We apologize to all those scientists whose papers could not be cited here due to the need of keeping this review within an appropriate size.

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